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(57) Abstract

A novel human E3 ubiquitin protein ligase is described. A structural region which encodes the polypeptide is disclosed as well as the amino acid residue sequence of the human ubiquitin protein ligase. Methods are provided to identify compounds that modulate the biological activity of the molecule and hence regulate cellular and tissue physiology.

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PROTEIN

Applicants herein claim priority from the U.S. Provisional Application, HUMAN E3 UBIQUITIN PROTEIN LIGASE, Serial No.60/073,839, filed February 5, 1998, as well as U.S. Application Serial No.09/070,060, filed April 30, 1998, each of which is incorporated by reference.

FIELD OF THE INVENTION

The present invention relates to nucleic acid and amino acid sequences of a novel human E3 ubiquitin protein ligase and to the use of these sequences to identify compounds that modulate the biological activity of the native biomolecule as well as modulate protein degradation or selective proteolysis and/or otherwise modulate physiological conditions associated with aberrant ubiquitin dependent proteolysis in human physiology. The invention is also related to the diagnosis, study, prevention, and treatment of pathophysiological disorders related to or mediated by the novel human E3 ubiquitin protein ligase.

BACKGROUND OF THE INVENTION

Three major proteolytic pathways (lysosomal, calcium-dependent, and the ATP-dependent pathways) exist in eukaryotic cells. The ATP-dependent pathway has long been known orchestrate specific degradation of native proteins. Recently it has become clear that the ATP-dependent ubiquitin mediated intracellular pathway is responsible for selective degradation of intact biomolecules as an efficiently evolved mechanism to adapt cellular physiology to the needs of the organism. Proteolysis is a powerful means of regulation due to the speed and irreversibility which enables the cell to rapidly eliminate or reduce the functional level of a particular biological molecule. *See, e.g.,* Jentsch, S., *et al., Selective Protein Degradation: A Journey's end Within the Proteasome,* Cell, 82:129 (1995). The critical role of ubiquitin-dependent proteolysis has steadily become increasingly clear, for example, in the normal degradation of oncoproteins and tumor suppressers in cell cycle control as well as in stress response and the immune system. Hochstrasser, M., Current Biology, 4:1024 (1992); Deshaies, R. J., Trends Cell Biol., 5:428 (1995); Hilt, W., *et al.,* Trends Biol. Sci., 21:96 (1996).

Ubiquitin is a heat-stable 76-amino acid biomolecule considered to be the most highly conserved protein known. Selective protein degradation *via* the ubiquitin pathway generally involves tagging of the target protein (substrate) by covalent attachment of multiple molecules of ubiquitin, and degradation of the target by the 26 S proteasome complex. Proteins are marked for direction to the proteasome *via* the covalent addition of branched polyubiquitin chains to the α-amino group of one or more surface lysines. The amide linkage of ubiquitin to a substrate protein is generally carried out by three classes of accessory enzymes in a sequential reaction. Ubiquitin activating enzymes (E1) activate ubiquitin by forming a high energy thiol ester intermediate. Activation of the C-terminal Gly of ubiquitin by E1, is followed by the activity of a ubiquitin conjugating enzyme E2 which serves as a carrier of the activated thiol ester form of ubiquitin during the transfer of ubiquitin directly to the third enzyme, E3 ubiquitin protein ligase. E3 ubiquitin protein ligase is responsible for the final step in the conjugation process which results in the formation of an isopeptide bond between the activated Gly residue of ubiquitin, and an α-NH group of a Lys residue in the substrate or a previously conjugated ubiquitin moiety. *See, e.g.,* Hochstrasser, M., *Ubiquitin-Dependent Protein Degradation*, Annu. Rev. Genet., 30:405 (1996).

In a reconstituted system, for example, all three categories of affinity purified enzymes (E1, E2, and E3) are required for the breakdown of ^{125}I-albumin to acid-soluble material in the presence of ubiquitin and ATP. Sears, C., *et al., NF-kB p105 Processing Via the Ubiquitin-Proteasome Pathway*, J Biol Chem., 273:1409 (1998). The high specificity of the ubiquitin selective-destruction pathway is predicted to allow the development of new classes of highly potent and selective low molecular weight enzyme inhibitors targeting particular members of the ubiquitin pathway that control the intracellular levels of a wide range of important regulatory proteins. Rolfe, M., *et al., The Ubiquitin-Mediated Proteolytic Pathway as a Therapeutic Area*, J. Mol. Med. 75:5-17 (1997).

Compelling evidence has been presented that implicates ubiquitination in the turnover of the tumor supressor protein, p53, cell cycle regulators cyclin A and cyclin B, the kinase c-mos, the cystic fibrosis transmembrane conductance regulator, the DNA repair protein O^6-methylguanine-DNA methyl transferase, the transcriptional co-activator p300, the

transcription factors c-jun, c-fos, IκB/NFκB, the transcription factors c-myc, DP1, and E2F, the regulatory subunit of cAMP-dependent protein kinase, receptors for peptide growth factors, estradiol receptor, as well as oncoprotein E1A. Moreover, as a corollary, pharmacological intervention which alters the half-lives of these cellular proteins is expected to have significant value in wide therapeutic potential, particularly in the areas of autoimmune disease, inflammation, cancer, as well as other proliferative disorders. Rolfe, M., *et al., The Ubiquitin-Mediated Proteolytic Pathway as a Therapeutic Area*, J. Mol. Med., 75:5 (1997).

E3 ubiquitin protein ligase, as the final player in the ubiquitination process, is responsible for target specificity of ubiquitin-dependent proteolysis. A number of E3 ubiquitin-protein ligases have previously been identified. *See, e.g.*, D'Andrea, A.D., *et al.*, Nature Genetics, 18:97 (1998); Gonen, H., *et al., Isolation, Characterization, and Purification of a Novel Ubiquitin-Protein Ligase, E3 - Targeting of Protein Substrates via Multiple and Distinct Recognition Signals and Conjugating Enzymes*, J. Biol. Chem., 271:302 (1996); Scheffner, M., *et al., The HPV-16 E6 and E6-AP Complex Functions as a Ubiquitin-Protein Ligase in the Ubiquitination of p53*, Cell, 75:495 (1993); Huibregtse, J.M., *et al., A Family of Proteins Structurally and Functionally Related to the E6-AP Ubiquitin Protein Ligase*, PNAS, 92:2563 (1995); Staub, O., *et al., WW Domains of Nedd4 Bind to the Proline-Rich PY Motifs in the Epithelial Na+ Channel Deleted in Liddles Syndrome*, EMBO, 15:2371 (1996) [the substrate specificity is determined by the E3 ligase]; Siepmann, T.J., *et al., Evidence for Stable, Exchangeable E1/E2/E3 Ubiquitin Conjugation Complexes at Physiological Concentrations*, FASEB J., 10:2324 (1996).

Other E3 ligases have been extensively evaluated in *S. cerevisiae* and in cell-free systems using engineered proteins as test substrates. Weissman, A. M., *Regulating Protein Degradation by Ubiquitination, Review Immunology Today*, 18(4):189 (1997); Sudakin, V., *et al.*, Mol. Biol. Cell, 6:185 (1995); Stancovski, I., *et al.*, Mol. Cell. Biol., 15:7106 (1995); King, R.W., *et al.*, Cell, 81:279 (1995); Chen, Z.J., *et al.*, Cell, 84:853 (1996); Orian, A., *et al.*, J. Biol. Chem., 170:21707 (1995); Varshavsky, A., *et al.*, Cell, 69:725 (1992); Hershko, A., *et al.*, Annu. Rev. Biochem., 61:761 (1992); Ciechanover, A., Cell, 7:13 (1994).

Perry *et al.*, recently identified a single gene which encodes a murine E3 ubiquitin protein ligase of the Hect family, disruption of which is demonstrated to cause an inflammatory phenotype of the mouse as well as enhanced epithelial and haematopoietic cell growth. Perry, W. L., *et al.*, Nature Genetics, 18:143 (1998). The murine E3 results reported by Perry *et al* indicate the specific ubiquitin-dependent proteolysis is an important mediator in the immune response as well as haematopoietic cell growth *in vivo*. Moreover, it is recently set forth that modulators of the E3 ubiquitin protein ligase are likely to have significant therapeutic potential, *inter alia*, as novel anti-inflammatory agents as well as entities to promote wound-healing. D'Andrea, A.D., *et al.*, Nature Genetics, 18:97 (1998); Perry, W. L., *et al.*, Nature Genetics, 18:143 (1998).

However, the previously reported E3 ubiquitin protein ligase is a murine isolate. The availability of an analogous functional human homolog will be ideal for the identification of compounds which modulate the specific biological activity of the E3 protein ligase and, as a corollary, modulate the physiological conditions associated with aberrant ubiquitin dependent proteolysis in human physiology. The availability of an analogous functional human homolog will also be ideal for the diagnosis, study, prevention, and treatment of pathophysiological disorders related to the biological molecule.

SUMMARY OF THE INVENTION

The present invention is directed to an isolated and purified polynucleotide molecule, which encodes a human E3 ubiquitin protein ligase, or a biologically-effective fragment thereof comprising a nucleic acid sequence encoding the polypeptide having the sequence substantially as depicted in SEQ ID NO:3 or a pharmacologically acive fragment thereof. Isolated and purified polynucleotides of the present invention include but are not limited to SEQ ID NO:1 (human E3 ubiquitin protein ligase cDNA) and SEQ ID NO:2 (human E3 ubiquitin protein ligase structural coding region).

In addition, the current invention is directed to a purified polypeptide comprising the amino acid sequence substantially as depicted in SEQ ID NO:3.

The invention is further directed to a host cell containing an expression vector for expression of a human E3 ubiquitin protein ligase polypeptide, wherein said vector contains a polynucleotide comprising a nucleic acid sequence encoding the polypeptide of a

human E3 ubiquitin protein ligase having the sequence substantially as depicted in SEQ ID NO:3 or a pharmacologically active fragment thereof. The invention is also directed to a method for producing a human E3 ubiquitin protein ligase polypeptide having the amino acid sequence substantially as depicted in SEQ ID NO:3 by culturing said host cell under conditions suitable for the expression of said polypeptide, and recovering said polypeptide from the host cell culture.

The instant invention is further directed to a method of identifying compounds that modulate the biological activity of a human E3 ubiquitin protein ligase, comprising:

(a) combining a candidate compound modulator of human E3 ubiquitin protein ligase biological activity with a human E3 ubiquitin protein ligase polypeptide having the sequence substantially as depicted in SEQ ID NO:3, and

(b) measuring an effect of the candidate compound modulator on the biological activity.

The instant invention is further directed to a method of identifying compounds that modulate the pharmacological activity of a human E3 ubiquitin protein ligase, comprising:

(a) combining a candidate compound modulator of human E3 ubiquitin protein ligase pharmacological activity with a host-cell expressing a human E3 ubiquitin protein ligase polypeptide having the sequence substantially as depicted in SEQ ID NO:3, and

(b) measuring an effect of the candidate compound modulator on the pharmacological activity.

The present invention is also directed to active compounds identified by means of the aforementioned methods, wherein said compounds modulate the biological and/or pharmacological activity of a human E3 ubiquitin protein ligase.

The invention is further directed to an antisense polynucleotide molecule comprising substantially the complement of SEQ ID NO:1 or a biologically-effective portion thereof, or SEQ ID NO:2 or a biologically-effective portion thereof, as well as a method for inhibiting the expression of a human E3 ubiquitin protein ligase comprising administering an effective amount of the antisense molecule.

The current invention is also drawn toward an antibody specific for a purified polypeptide comprising the amino acid sequence substantially as depicted in SEQ ID NO:3, as well as a

diagnostic composition for the identification of a polypeptide sequence comprising the amino acid sequence substantially as depicted in SEQ ID NO:3.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 displays SEQ ID NO:1 which is a 5372 base cDNA nucleic acid sequence which encodes the novel human E3 ubiquitin protein ligase described herein.

Figure 2 displays SEQ ID NO:2 which is a 2559 base translated structural coding region, ATG to TAA (*Ochre*), of the cDNA nucleic acid sequence which encodes the novel human E3 ubiquitin protein ligase (human homolog of the murine *itchy* locus; Perry, W.L., *et al.*, Nature Genetics, 18:143 (1998)).

Figure 3 displays SEQ ID NO:3 which is a 852 amino acid residue sequence of the human E3 ubiquitin protein ligase homolog described herein.

Figure 4 shows SEQ ID NO:4 which is the 854 amino acid residue sequence of the murine E3 ubiquitin protein ligase (mapped to *itchy* locus). Perry, W. L., *et al.*, Nature Genetics, 18:143 (1998); Hustad, C. M., *et al.*, Genetics, 140:255 (1995).

Figure 5 displays a comparison alignment between the amino acid residue sequence of the novel human E3 ubiquitin protein ligase homolog described herein (SEQ ID NO:3), and the amino acid residue sequences of the murine E3 ubiquitin protein ligase (SEQ ID NO:4).

Figure 6 displays Northern blot analyses of multiple tissues using a nucleic acid probe specific to the human E3 ubiquitin protein ligase coding region described herein (SEQ ID NO:2). Key : tracks 1-14 represent pancreas, kidney, skeletal muscle, liver, lung, placenta, brain, heart, fetal liver, bone marrow, PBL, thymus, lymph node and spleen respectively.

Figure 7 displays PCR primers, SEQ ID NO:5 (sense) and SEQ ID NO:6 (antisense), which can be used to amplify the 2559 bp coding region (SEQ ID NO:2) of the novel human E3 ubiquitin protein ligase from human tissue.

Figure 8 shows a schematic representation of example Scintillation Proximity Assays (SPA), as well as RIA and ELISA Assays.

Figure 9 displays SEQ ID NO:7 which is the 156 amino acid precursor peptide to the mature 76 amino acid residue sequence of human ubiquitin. Lund P.K., *et al.*, J. Biol. Chem., 260:7609 (1985).

Figure 10 displays SEQ ID NO:8 which is the mature 76 amino acid residue sequence of native human ubiquitin (positions 1-76 of SEQ ID NO:7). Lund P.K., *et al.*, J. Biol. Chem., 260:7609 (1985).

Figure 11 displays SEQ ID NO:9 which is the 471 base translated structural coding region, ATG to TAA (*Ochre*), of the cDNA nucleic acid sequence which encodes the 156 amino acid precursor peptide (SEQ ID NO:7) to the mature 76 amino acid residue sequence of native human ubiquitin (positions 1-76 of SEQ ID NO:7). Lund P.K., *et al.*, J. Biol. Chem., 260:7609 (1985).

Figure 12 displays SEQ ID NO:10 which is the 3177 base translated structural coding region, ATG to TGA (*Opal*), of the nucleic acid sequence which encodes the previously described 1058 amino acid residue human E1 ubiquitin activating enzyme (Uba1). Ayusawa, D., *et al.*, Cell Struct. Funct., 17:113 (1992).

Figure 13 displays SEQ ID NO:11 which is the 444 base translated structural coding region, ATG to TGA (*Opal*), of the nucleic acid sequence which encodes the previously described 147 amino acid residue E2 ubiquitin conjugating enzyme $E2_{17k}$ (Ub10a). Wing S.S., *et al.*, Biochem. J., 305:125 (1995). The human version (Ubc2) is preferred as described by Koken, M., *et al.*, PNAS, 88:8865 (1991).

Figure 14 demonstrates the recombinant human E3 ubiquitin ligase (SEQ ID NO:3) has ubiquitinating activity *in vitro*.

Figure 15 demonstrates a dominant negative mutant version of SEQ ID NO:3 (C820A) has no enzymatic activity (lane A) (ubiquitination) compared to the wild type control (lane B).

Figure 16 demonstrates a marked decline in the intracellular SEQ ID NO:3 levels 2 h after activation of the jurkat T cells by PMA and ionomycin in Western blot analysis. The arrow designates a band pertaining to SEQ ID NO:3.

Figure 17 demonstrates a dramatic decline in the level of human itchy E3 ligase mRNA within 3 h after stimulation of PBMC's. The top panel represents a Northern blot (arrow designates a band pertaining to SEQ ID NO:1); the botttom panel represents the corresponding ethidium bromide stained gel.

DETAILED DESCRIPTION OF THE INVENTION

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. All publications and patents referred to herein are incorporated by reference.

Nucleic acid sequence as used herein refers to an oligonucleotide, nucleotide or polynucleotide sequence, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which may be double-stranded or single-stranded whether representing the sense or antisense strand. Similarly, amino acid and/or residue sequence as used herein refers to peptide or protein sequences or portions thereof.

Biological activity as used herein refers to the ability of the novel human E3 ubiquitin protein ligase and variations thereof contemplated herein to bind ubiquitin and/or transfer ubiquitin to a substrate under biological conditions.

Pharmacological activity, as used herein in reference to the novel human E3 ubiquitin protein ligase and variations thereof contemplated herein, refers to the ability to modulate protein degradation or selective proteolysis and/or otherwise modulate physiological conditions associated with aberrant ubiquitin dependent proteolysis in human physiology.

Dominant negative mutant as used herein refer to a nucleic acid coding region sequence which has been changed with regard to at least one position in the sequence, relative to the corresponding wild type native version, preferrably at a position which changes an amino acid residue position at an active site required for biological and/or pharmacological activity in the native peptide to thereby encode a mutant peptide.

The term 'modulation' is used herein to refer to the capacity to either enhance or inhibit the biological activity of a E3 ubiquitin protein ligase. The term "modulation" is also used herein to refer to the pharmacological capacity to to either enhance or inhibit the selective elimination of a biological protein molecule *via* ubiquitin dependent proteolysis under biological conditions.

Purified as used herein refers to molecules, either nucleic acid or amino acid sequences, that are removed from their natural environment and isolated or separated from at least one other component with which they are naturally associated.

As used herein, a functional derivative of a biomolecule disclosed herein is an entity that possesses a functional biological activity and/or pharmacological activity as defined herein that is derived from SEQ ID NO:1 or SEQ ID NO:3, for example, truncated versions, versions having deletions, functional fragments, versions having substitutions, versions having insertions or extended ends, or biologically effective dominant negative mutants as well as biologically effective antisense molecules.

Substantially as depicted as used herein refers to functional derivative proteins, and functional derivative nucleic acid sequences as defined herein that may have changes but perform substantially the same biochemical or pharmacological function in substantially the same way; however, 'substantially as depicted' as used herein also refers to biologically effective dominant negative mutants and is intended to encompass biologically effective antisense molecules as defined herein.

Biologically effective as used herein in reference to antisense nucleic acid molecules as well as dominant negative mutant nucleic acid coding regions and dominant negative mutant peptides refers to the ability of these molecules to modulate the biological activity and/or pharmacological activity of the novel signal transduction protein kinase of the present invention and/or transcription/translation of nucleic acid coding regions of the novel signal transduction protein kinase of the present invention.

Expression vector as used herein refers to nucleic acid vector constructions which have components to direct the expression of heterologous protein coding regions including coding regions of the present invention through accurate transcription and translation in host cells. Expression vectors usually contain a promoter to direct polymerases to transcribe the heterologous coding region, a cloning site at which to introduce the heterologous coding region, and usually polyadenylation signals. Expression vectors include but are not limited to plasmids, retroviral vectors, viral and synthetic vectors.

Transformed host cells as used herein refer to cells which have coding regions of the present invention stably integrated into their genome, or episomally present as replicating or nonreplicating entities in the form of linear nucleic acid or transcript or circular plasmid or vector. Transformation or transformed as used herein refers to heterologous gene expression including but not limited to transient or stable transfection systems.

Direct administration as used herein refers to the direct administration of nucleic acid constructs which encode embodiments (e.g., SEQ ID NO:3, dominant/negative mutant version, antisense molecule, antibody molecule, modulator compound molecule) of the present invention or fragments thereof; and the direct administration of embodiments of the present invention or fragments thereof, and the *in vivo* introduction of molecules of the present invention preferably via an effective eukaryotic expression vector in a suitable pharmaceutical carrier. Polynucleotides and therapeutic molecules of the present invention may also be delivered in the form of nucleic acid transcripts.

Ubiquitin-dependent Proteolysis

Ubiquitination has recently become a focal point in cell biology as it is acknowledged in joining phosphorylation as a major protein modification device in regulation of cell physiology. The importance of ubiquitin-dependent proteolysis for selective elimination of biomolecules is indisputable for the maintenance of cellular integrity and physiology of the organism. The depth of current knowledge about the molecular mechanisms regulating ubiquitin-dependent proteolysis, combined with the understanding of how impairment of such processes, underlies pathological conditions, has opened the way for a mechanism-based approach for the development of new drugs. Rolfe, M., *et al., The Ubiquitin-Mediated Proteolytic Pathway as a Therapeutic Area*, J. Mol. Med. 75:5-17 (1997).

A growing number of cellular regulatory mechanisms are being linked to ubiquitin. For instance, ubiquitination is a widely utilized ligand-mediated means of modulating transmembrane receptor function. Mammalian transmembrane receptors found to undergo ligand-mediated ubiquitination are coupled to, or are themselves, tyrosine kinases. Ubiquitination of the T-cell receptor (TCR) is stimulated by antigen (MHC and peptide), superantigens, lectins that bind the TCR, or by anti-receptor antibodies. Moreover, correlations between dysregulated ubiquitination/proteasomal degradation and cellular transformation are striking. Weissman, A. M., *Regulating Protein Degradation by Ubiquitination, Review Immunology Today*, 18(4):189 (1997).

Ubiquitination is now implicated in regulating numerous cellular processes including: signal transduction, cell-cycle progression, receptor-mediated endocytosis, transcription (including activation-induced transcription in lymphocytes), organelle biogenesis and

spermatogenesis. Abnormal accumulations of ubiquitinated species are found in intracellular inclusions in neuropathological conditions including Alzheimer's and Pick's diseases. The importance of regulated ubiquitination is demonstrated by the resistance of oncogenic counterparts of normal cellular ubiquitination substrates to this post-translational modification, and by correlations between malignant transformation and loss of function, or dysregulated function, of enzymes involved in ubiquitination. Proteasomes have recently been implicated in programmed cell death in neurons and thymocytes at points proximinal to activation of the interleukin-1β-converting enzyme (ICE) family of proteases. Morover, dysregulated ubiquitination contributes to malignant transformation, for example, oncogenic counterparts of normally ubiquitinated proteins are resistant to ubiquitination. Weissman, A. M., *Regulating Protein Degradation by Ubiquitination, Review Immunology Today*, 18(4):189 (1997); Papavassiliou, A.G., *et al.*, Science, 258:1941 (1992); Treier, M., *et al.*, Cell, 78:787 (1994); Papa, F.R., *et al.*, Nature, 366:319 (1993); Grimm, L. M., *et al.*, EMBO, 15:3835 (1996); Sadoul, R., *et al.*, EMBO, 15:3845 (1996).

E3 Ligase

Ubiquitin-dependent proteolysis needs to be very selective in order to effectively regulate intracellular physiology. The component of the ubiquitin conjugation system generally believed to be the most directly involved in substrate recognition is the E3 protein ligase. *See, e.g.*, Hochstrasser, M., *Ubiquitin-Dependent Protein Degradation*, Annu. Rev. Genet., 30:405 (1996). The first E3 ligase to be identified, E6-AP, is the best previously characterized member of the Hect-domain class. E6-AP was originally identified through its interaction with the E6 oncoprotein of the cancer-associated human papillomavirus types 16 and 18. The E6/E6-AP complex specifically binds to the tumor suppressor protein p53 and induces its ubiquitination and subsequent degradation. The cysteine residue necessary for thioester formation of E6-AP with ubiquitin is conserved among all of the Hect-domain class proteins. Because of this similarity these proteins have been termed Hect proteins, for 'Homologous to E6-AP C Terminus' (HECT).

An essential intermediate step in E6-AP-dependent protein ubiquitination is the formation of a thioester complex between ubiquitin and E6-AP. Furthermore, the direction of ubiquitin transfer is from E1 to E2 and then from E2 to E6-AP. This suggests that in this

particular system, the E3 catalyzes the final attachment of ubiquitin to a substrate protein, rather than the E2 as shown for few other systems. The cysteine residue of E6-AP involved in thioester formation has been mapped to the carboxyl terminus. The carboxyl-terminal regions of several proteins from different organisms show significant similarity to the carboxyl terminus of E6-AP.

Furthermore, another ubiquitin protein ligase (E3) has been characterized as the neuronal precursor cell-expressed developmentally downregulated 4 (Nedd4). The biological structure is a multimodular protein composed of a C2 domain, 3 (or 4) WW domains, and a C-terminal ubiquitin protein ligase Hect domain. Nedd4 is a protein that interacts with the epithelial Na+ channel (ENaC) which is mediated by an association of the WW domains of Nedd4 with the proline-rich PY motifs (XPPXY, where X = any amino acid) of the ENaC subunits. Deletion or mutations within the PY motifs of the ENaC subunits have been genetically linked to Liddle syndrome, a hereditary form of systemic renal hypertension caused by an abnormal increase in ENaC activity. Recent work has described interaction of Nedd4 and Nedd4-like proteins with other PY motif-containing proteins, also mediated by the WW domains wherein the substrate specificity is determined by the E3 ligase. Staub, O., *et al.*, EMBO, 15:2371 (1996).

Human E3 Ubiquitin Protein Ligase

The human E3 ligase described herein is a member of the Hect-domain containing ubiquitin-protein ligases, named for the highly conserved C-terminal portion of the molecule. SEQ ID NO:1 is a 5372 base cDNA nucleic acid sequence which encodes the novel human E3 ubiquitin protein ligase described herein (FIG.1). SEQ ID NO:2 is a 2559 base translated structural coding region of the cDNA nucleic acid sequence which encodes the novel human E3 ubiquitin protein ligase (human homolog of the murine 'itchy' locus; Perry, W.L., *et al.*, Nature Genetics, 18:143 (1998)). SEQ ID NO:3 is a 852 amino acid residue sequence of the human E3 ubiquitin protein ligase homolog described herein. SEQ ID NO:4, for comparison, is the 854 amino acid residue sequence of the murine E3 ubiquitin protein ligase mapped to 'itchy' locus as described by Perry, W. L., *et al.*, Nature Genetics, 18:143 (1998); Hustad, C. M., *et al.*, Genetics, 140:255 (1995).

A comparison alignment between the amino acid residue sequence of the novel human E3 ubiquitin protein ligase homolog described herein (SEQ ID NO:3), and the amino acid residue sequences of the murine E3 ubiquitin protein ligase (SEQ ID NO:4) is shown in FIG.5. The native human protein (SEQ ID NO:3) is 96% homologous to the murine 'itchy' E3 ubiquitin ligase (SEQ ID NO:4), at the amino acid level. The Hect class of E3 ligases contain 3 or 4 protein-protein interaction domains known as WW domains, named for two conserved tryptophan (W) residues. Sequence alignment and structural features of the human E3 ligase protein (SEQ ID NO:3) compared to the mouse 'itchy' protein (SEQ ID NO:4) demonstrates that both proteins share the approximately 350 AA HECT domain at the C terminus; and both molecules have 4 WW protein interaction domains. Particularly pertaining to the human E3 ubiquitin protein ligase → WWI: positions 275-306 of SEQ ID NO:3; WWII: positions 307-340 of SEQ ID NO:3; WWIII: positions 386-420 of SEQ ID NO:3; WWIV: positions 427-460 of SEQ ID NO:3. Furthermore, the conserved cysteine residue where a ubiquitin linkage is expected to occur is also apparent at position 820 of SEQ ID NO:3. This residue position is of particular significance especially for the construction of pharmacologically valuable dominant negative mutants. *See*, Example VIII. The novel human E3 recombinant enzyme described herein, e.g., SEQ ID NO:3, is expected to have inherently high native catalytic activity. Moreover, any change (e.g., substitution or deletion) to the residue where ubiquitin linkage is expected to occur (SEQ ID NO:3, cysteine position 820) is expected to be significantly compromise the catalytic activity of the native human E3 ubiquitin protein ligase.

The 'itchy' mice (murine E3 ligase knockout) display a phenotype that suggests activation of processes typical of chronic inflammatory and/or wound healing events, including lymphoid hyperplasia and hematopoietic cell proliferation further discussed *infra*. In order to evaluate the role of SEQ ID NO:3 E3 ligase in the activation of human leukocytes, stimulation experiments were performed independently using peripheral blood mononuclear cells, Jurkat cells, and U937 cells. *See*, Example IX. Following stimulation, cells were collected by low-speed centrifugation and lysed to isolate either protein for Western blot analysis (FIG.16) or RNA for Northern blot analysis (FIG.17). Proteins were transferred to nitrocellulose membranes, immunoblotted using an anti-peptide antibodies

described herein and a horseradish peroxidase-conjugated anti-rabbit secondary antibody. A marked decline is demonstrated in the intracellular SEQ ID NO:3 levels 2 h after activation of the jurkat T cells. Activation of T lymphocytes by PMA and ionomycin results in a signal transduction cascade; the findings demonstrated herein indicate that the SEQ ID NO:3 'itchy' E3 ligase is involved in turnover of signal transduction proteins in the lymphocytic cells. *See*, FIG.16. The results demonstrate that the human itchy E3 ligase mRNA levels dramatically decline within 3 h after stimulation of PBMC's. These results suggest that the 'itchy' E3 ligase gene is involved in turnover of signal transduction molecules in the hematopoietic lineages. *See*, FIG.17.

Chromosomal Location

The E3 ligase gene (SEQ ID NO:1) has been mapped to human chromosome 20q11.23-12 using the Stanford G3 radiation hybrid panel. The most proximal markers are SHGC 53176, SHGC 8755, and SHGC 2765.

Substrate

The novel human E3 ubiquitin protein ligase (e.g., SEQ ID NO:3) ubiquitinates specific intracellular biological molecules *in vivo* to effect selective destruction and swift regulation of cellular physiology. Biological activity refers to the ability of the novel human E3 ubiquitin protein ligase and variations thereof contemplated herein to bind ubiquitin and/or transfer ubiquitin to a substrate under biological conditions. Substrates include the likes of intracellular messenger biological molecules, receptors, ligands, signal transduction molecules, transcriptional activators, cytokines, kinases, phosphatases and phosphorylases, especially which mediate physiological conditions such as inflammation, autoimmune disease, neurological disease, apoptosis, endothelial cell physiology (e.g., proliferation, differentiation), peripheral vascular disease, angiogenesis, cancer, anemia, hematopoietic disorders, cachexia, leukemia, pulmonary disorders, arthritis, diabetes, and viral infection. Pharmacological activity, as used herein in reference to the novel human E3 ubiquitin protein ligase (e.g., SEQ ID NO:3) and variations thereof contemplated herein, refers to the ability to modulate protein degradation or selective proteolysis and/or otherwise modulate physiological conditions associated with aberrant ubiquitin dependent proteolysis in human

physiology (e.g., disorders manifested by aberrant forms and/or abnormal levels of the native E3 ubiquitin protein ligase).

Pharmacological Significance

The control of hematopoiesis is a highly regulated process that responds to a number of physiological stimuli in the human body. Differentiation, proliferation, growth arrest, or apoptosis of blood cells depends on the presence of appropriate cytokines and their receptors, as well as the corresponding cellular signal transduction cascades. Hu, Mickey C.-T., *et al.*, Genes & Development, 10:2251(1996). Generation of mature leukocytes, for instance, is a highly regulated process which responds to various environmental and physiological stimuli. Cytokines cause cell proliferation, differentiation or elimination, each of these processes being dependent on the presence of appropriate cytokine receptors and the corresponding signal transduction elements. Moreover, the stimulation of quiescent B- and T-lymphocytes occur *via* antigen receptors which exhibit remarkable homology to cytokine receptors. Grunicke, Hans H., *Signal Transduction Mechanisms in Cancer*, Springer-Verlag (1995). See also, Suchard, S.J., *et al.*, *Mitogen-Activated Protein Kinase Activation During IgG-Dependent Phagocytosis in Human Neutrophils*, J. Immunol., 158:4961 (1997).

The identification of a single gene underlying an inflammatory syndrome provides significant potential to identify novel targets for anti-inflammatory drugs, *inter alia*. Modulators of the human E3 ubiquitin protein ligase described herein accordingly have significant potential as novel anti-inflammatory agents as well as agents to promote wound-healing. *See*, D'Andrea, A.D., *et al.*, Nature Genetics, 18:97 (1998). Moreover, compounds which modulate the biological activity of the human E3 ubiquitin protein ligase *in vivo* are expected to to influence hematopoiesis. The 'itchy' knockout mice (murine E3 ubiquitin protein ligase (SEQ ID NO:4)) have demonstrated enhanced hematopoiesis, manifested, for example, by accelerated development of the erythroid, myeloid, and lymphoid lineages. The homozygous mouse has been demonstrated to exhibit an apparent pan-hematopoiesis, resulting in the accumulation of inflammatory cells in organs and the skin and a macrophage infiltrate in the lung. The C57BL/6J 'itchy' mice have a phenotype that suggests activation of processes typical of chronic inflammatory and/or wound healing

events, including lymphoid hyperplasia, hematopoietic cell proliferation and gastorintestinal epithelial hyperplasia. The mice also display chronic inflammation of airways, skin and stomach. The 'itchy' E3 ligase appears to mediate the turnover of signal transduction proteins in the hematopoietic lineages.

The murine E3 ligase (SEQ ID NO:4), involved in ubiquitin-mediated protein degradation, is believed to specifically mediate the turnover of growth factor signal transduction proteins in the hematopoietic lineages. By analogy, the human homolog E3 ubiquitin protein ligase described herein (SEQ ID NO:3) is expected to likewise significantly influence hematopoiesis. Moreover, results indicate that ubiquitin-dependent proteolysis is an important mediator of the immune response *in vivo* and provides evidence for the 'itchy' E3's role in inflammation and the regulation of epithelial and haematopoietic cell growth. Perry, W. L., *et al.*, Nature Genet., 18:143 (1998); Rolfe, M., *et al., The Ubiquitin-Mediated Proteolytic Pathway as a Therapeutic Area*, J. Mol. Med. 75:5-17 (1997). Accordingly, specific modulation of the biological and/or pharmacological activity of the human "itchy" E3 ligase, e.g., SEQ ID NO:3, *via* administration of a compound modulator or heterologous expression or administration of a dominant negative mutant version or antisense molecule derived from SEQ ID NO:1 is expected to significantly influence inflammation as well as hematopoiesis. Modulation of the biological and/or pharmacological activity of the human 'itchy' (e.g., SEQ ID NO:3) is expected to modulate lymphocyte function, for example by inhibiting human 'itchy' activity, resulting in anti-tumor activity. Modulation of the human E3 ligase activity is moreover contemplated in applications for supportive hematopoietic therapy; for example, in subjects wherein cancer therapy impairs bone marrow function or in immune suppressed cancer patients. Inhibition of the biological activity of 'itchy' (SEQ ID NO:3) is expected to accelerate development of the erythroid, myeloid, and lymphoid lineages. Blockage or reduction of 'itchy' (e.g., SEQ ID NO:3) activity by a compound or other pharmacologic agent is expected to stimulate hematopoiesis as well as expansion of activated lymphocytes (e.g., expand T lymphocytes in cancer patients).

Example human E3 ubiquitin protein ligase substrates include, but are not limited to, GM-CSF and its receptor, G-CSF and its receptor, SCF and its receptor c-kit, IL-3 and IL-3r, IL-5 and IL-5r, and IL-6 and IL-6r.

Hematopoiesis can be severely compromised by cytotoxic chemotherapy and irradiation. High-dose conditioning therapies that include total body irradiation, for instance, are notably myelotoxic and require the transplantation of hematopoietic progenitor cells. *See, e.g.*, Thomas, E. D., *et al.*, N. Engl. J. Med., 25:491 (1987); Berenson, R. J. *et al.*, Blood. 77:1717 (1991). Such adoptive cellular immunotherapy is regularly accompanied by growth factor administration, e.g., erythropoietin (Epogen), G-CSF (Neupogen), GM-CSF, and thrombopoietin in respective therapeutic applications. Modulators of the novel human E3 ubiquitin protein ligase as described herein are therefore contemplated as therapeutic agents to compete with the likes of erythropoietin (Epogen), G-CSF (Neupogen), and thrombopoietin in the respective applications. Applications are also contemplated for supportive hematopoietic care, including cancer therapies that impair bone marrow function and AIDS/HIV.

Cachexia is a condition characterized by severe muscle atrophy, weight loss and emaciation. Ubiquitin dependent proteolysis has been linked to the skeletal muscle loss during cachexia as well in in tumors. Medina, R., *et al.*, Biomed. Biochim. Acta. 50:4 (1991); Temparis, S., et al., Cancer Research, 54: 5568 (1994); Tiao, G. *et al.*, J. Clin. Invest., 94:2255 (1994). Furthermore, ubiquitin dependent proteolysis has recently been implicated in the down regulation of signal transducing receptors. Particularly the involvement of the ubiquitin conjugation system in the ligand induced endocytosis and degradation of the growth hormone receptor may be of particular importance in cachexia conditions. The b2-Adrenergic Agonist, Clenbuterol, for instance, has been demonstrated to prevent enhanced muscle protein degradation, and that normalization of protein breakdown is achieved through a decrease of the hyperactivation of the ubiquitin dependent proteolysis system. Costelli, P., *et al.*, J. Clin. Invest 95:2367 (1995).

The novel human E3 recombinant enzyme described herein, e.g., SEQ ID NO:3, is expected to have inherently high native catalytic activity. Clearly defined biological activity permits easy adaptation of the ligase to methods for identifying compounds that

modulate the biological and/or pharmacological activity of the novel human E3 ubiquitin protein ligase and variations thereof contemplated herein, for instance, *via* automated high throughput biochemical assays, e.g., scintillation proximity assays, further described infra. For instance, a specific low molecular weight inhibitor of ubiquitin transfer onto cyclin B, targeting the E3 involved in this process, would prevent cyclin B destruction and would be expected to be a very strong cytostatic agent. Rolfe, M., *et al., The Ubiquitin-Mediated Proteolytic Pathway as a Therapeutic Area*, J. Mol. Med. 75:5-17 (1997). Accordingly, specific modulation of the biological and/or pharmacological activity of the novel human E3 ubiquitin protein ligase, e.g., SEQ ID NO:3, *via* administration of a compound modulator or heterologous expression or administration of a dominant negative mutant version is expected to have a high degree of biological specificity for the treatment of physiological conditions including, but not limited to, inflammation, autoimmune disease, neurological disease, apoptosis, endothelial cell physiology (e.g., proliferation, differentiation), peripheral vascular disease, angiogenesis, cancer, anemia, hematopoietic disorders, cachexia, leukemia, pulmonary disorders, arthritis, diabetes, and viral infection.

Variants

The present invention also encompasses variants of the human E3 ubiquitin protein ligase SEQ ID NO:3. A variant substantially as depicted in SEQ ID NO:3, for instance, is one having 98% total amino acid sequence similarity to the human E3 ubiquitin protein ligase amino acid sequence (SEQ ID NO:3) or a biologically active fragment thereof. A preferred variant substantially as depicted in SEQ ID NO:3 is one which retains at least one of the amino acid residues which are characteristic of the human homolog E3 ubiquitin protein ligase described herein.

A "variant" of the human E3 ubiquitin protein ligase molecule of the present invention may have an amino acid sequence that is different by one or more amine acid "substitutions". The variant may have "conservative" changes, wherein a substituted amine acid has similar structural or chemical properties, eg, replacement of leucine with isoleucine. More rarely, a variant may have "nonconservative" changes, eg, replacement of a glycine with a tryptophan. Similar minor variations may also include amine acid deletions or insertions, or both. Guidance in determining which and how many amine acid residues may be

substituted, inserted or deleted without abolishing biological or immunological activity, for instance, may be found using computer programs well known in the art, for example, DNAStar software.

The present invention relates to nucleic acid (SEQ ID NO:1 and SEQ ID NO:2) and amino acid sequences (SEQ ID NO:3) of the novel human E3 ubiquitin protein ligase and variations thereof and to the use of these sequences to identify compounds that modulate the activity of E3 ubiquitin protein ligase under biological conditions as well as human physiology.

The invention further relates to the use of the nucleic acid sequences described herein in expression systems as assays for agonists or antagonists of the E3 ubiquitin protein ligase. The invention also relates to the diagnosis, study, prevention, and treatment of disease related to the human E3 ubiquitin protein ligase and/or diseases mediated by the biomolecule.

Polynucleotide sequences which encode the human E3 ubiquitin protein ligase(SEQ ID NO:3) or a functionally equivalent derivative thereof may be used in accordance with the present invention which comprise deletions, insertions and/or substitutions of the SEQ ID NO:2 nucleic acid sequence. Biologically active variants of the biomolecule of the present invention may also be comprised of deletions, insertions or substitutions of SEQ ID NO:3 amino acid residues. A purified polynucleotide comprising a nucleic acid sequence encoding the polypeptide having the sequence substantially as depicted in SEQ ID NO:3 or a biologically active fragment thereof is a particularly preferred embodiment of the present invention.

Amino acid substitutions of SEQ ID NO:3 may be made, for instance, on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the pharmacological or biological activity of the E3 ubiquitin protein ligase is retained.

Dominant/negative mutants are also contemplated wherein codons for one or more known functional residues are deleted or changed in the coding region (e.g., SEQ ID NO:2) in order to encode a mutant variation having valuable pharmacological function. For example, characteristic residues for ubiquitin transfer (e.g., *the conserved cysteine residue*

at SEQ ID NO:3 position 820 where a ubiquitin linkage is expected to occur) may be changed or deleted. *See*, Example VIII. Methods of treatment of conditions manifested by aberrant forms and/or abnormal levels of the native E3 ubiquitin protein ligase *via* administration of a polypeptide substantially as depicted in SEQ ID NO:3 or a pharmacologically active fragment thereof, or a nucleic acid substantially as depicted in SEQ ID NO:1, as referred to herein, is defined to encompass dominant/negative mutant versions of these entities.

For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine phenylalanine, and tyrosine.

Nucleic acid sequences which encode the amino acid sequence of the human ubiquitin ligase described herein are of an exponential sum due to the potential substitution of degenerate codons (different codons which encode the same amino acid). The oligonucleotide sequence selected for heterologous expression is therefore preferably tailored to meet the most common characteristic tRNA codon recognition of the particular host expression system used as well known by those skilled in the art.

Suitable conservative substitutions of amino acids are known to those of skill in this art and may be made without altering the biological activity of the resulting polypeptide, regardless of the chosen method of synthesis. The phrase "conservative substitution" includes the use of a chemically derivatized residue in place of a non-derivatized residue provided that such polypeptide displays the desired binding activity. D-isomers as well as other known derivatives may also be substituted for the naturally occurring amino acids. *See, e.g.*, U.S. Patent No. 5,652,369, *Amino Acid Derivatives*, issued July 29, 1997. Substitutions are preferably, although not exclusively, made in accordance with those set forth in TABLE 1 as follows:

TABLE 1

Original residue	Example conservative substitution
Ala (A)	Gly; Ser; Val; Leu; Ile; Pro
Arg (R)	Lys; His; Gln; Asn
Asn (N)	Gln; His; Lys; Arg
Asp (D)	Glu
Cys (C)	Ser
Gln (Q)	Asn
Glu (E)	Asp
Gly (G)	Ala; Pro
His (H)	Asn; Gln; Arg; Lys
Ile (I)	Leu; Val; Met; Ala; Phe
Leu (L)	Ile; Val; Met; Ala; Phe
Lys (K)	Arg; Gln; His; Asn
Met (M)	Leu; Tyr; Ile; Phe
Phe (F)	Met; Leu; Tyr; Val; Ile; Ala
Pro (P)	Ala; Gly
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr; Phe
Tyr (Y)	Trp; Phe; Thr; Ser
Val (V)	Ile; Leu; Met; Phe; Ala

The nucleotide sequences of the present invention may also be engineered in order to alter a coding sequence for a variety of reasons, including but not limited to, alterations which modify the cloning, processing and/or expression of the gene product. For example, mutations may be introduced using techniques which are well known in the art, eg, site-

directed mutagenesis to insert new restriction sites, to alter glycosylation patterns, to change codon preference, etc.

Included within the scope of the present invention are alleles of the human E3 ubiquitin protein ligase molecule of the present invention. As used herein, an "allele" or "allelic sequence" is an alternative form of the E3 ubiquitin protein ligase molecule described herein. Alleles result from nucleic acid mutations and mRNA splice-variants which produce polypeptides whose structure or function may or may not be altered. Any given gene may have none, one or many allelic forms. Common mutational changes which give rise to alleles are generally ascribed to natural deletions, additions or substitutions of amino acids. Each of these types of changes may occur alone, or in combination with the others, one or more times in a given sequence.

The present invention relates, in part, to the inclusion of the polynucleotide encoding the novel E3 ubiquitin protein ligase molecule in an expression vector which can be used to transform host cells or organisms. Such transgenic hosts are useful for the production of the novel selective degradation molecule and variations thereof described herein.

The nucleic acid sequence also provides for the design of antisense molecules useful in downregulating, diminishing, or eliminating expression of the genomic nucleotide sequence in cells including but not limited to hematopoietic, endothelial, and tumor or cancer cells.

The human E3 ubiquitin protein ligase biomolecule of the present invention can also be used in screening assays to identify blockers, antagonists or inhibitors which bind, emulate substrate, or otherwise inactivate or compete with the biomolecule. The novel E3 ubiquitin protein ligase can also be used in screening assays to identify agonists which activate the E3 ubiquitin ligase or otherwise induce the production of or prolong the lifespan of the biomolecule *in vivo* or *in vitro*.

The invention also relates to pharmaceutical compounds and compositions comprising the human E3 ubiquitin protein ligase molecule substantially as depicted in SEQ ID NO:3, or fragments thereof, antisense molecules capable of disrupting expression of the naturally occurring gene, and agonists, antibodies, antagonists or inhibitors of the native biomolecule.

Generally acceptable Vectors

In accordance with the present invention, polynucleotide sequences which encode the human E3 ubiquitin protein ligase polypeptide, fragments of the polypeptide, fusion proteins, or functional equivalents thereof may be used in recombinant DNA molecules that direct the expression of the ubiquitin ligase biomolecule in appropriate host cells. Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence, may be used to clone and express the human biomolecule as well as variations thereof contemplated herein. As will be understood by those of skill in the art, it may be advantageous to produce the human E3 ubiquitin ligase encoding nucleotide sequences which possess non-naturally occurring codons.

Specific initiation signals may also be required for efficient translation of an E3 ubiquitin ligase nucleic acid sequence. These signals include the ATG initiation codon and adjacent sequences. In cases where the human E3 ubiquitin ligase nucleic acid sequence, e.g., SEQ ID NO:2, its initiation codon and upstream sequences are inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only coding sequence, or a portion thereof, is inserted, exogenous transcriptional control signals including the ATG initiation codon must be provided. Furthermore, the initiation codon must be in the correct reading frame to ensure transcription of the entire insert. Exogenous transcriptional elements and initiation codons can be of various origins, both natural and synthetic.

Nucleic acid sequences, e.g., SEQ ID NO:2, may be recombinantly expressed to produce a pharmacologically active E3 ubiquitin ligase biomolecule by molecular cloning into an expression vector containing a suitable promoter and other appropriate transcription regulatory elements, and transferred into prokaryotic or eukaryotic host cells to produce the novel polypeptide. Techniques for such manipulations are, for instance, fully described in Sambrook, J., *et al.*, Molecular Cloning Second Edition, Cold Spring Harbor Press (1990), and are well known in the art.

Expression vectors are described herein as DNA sequences for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host cell. Such

vectors can be used to express nucleic acid sequences in a variety of hosts such as bacteria, bluegreen algae, plant cells, insect cells, fungal cells, human, and animal cells. Specifically designed vectors allow the shuttling of DNA between hosts such as bacteria-yeast, or bacteria-animal cells, or bacteria-fungal cells, or bacteria-invertebrate cells.

A variety of mammalian expression vectors may be used to express the recombinant E3 ubiquitin ligase molecule and variations thereof disclosed herein in mammalian cells. Commercially available mammalian expression vectors which are suitable for recombinant expression, include but are not limited to, pcDNA3 (Invitrogen), pMC1neo (Stratagene), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593) pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460), and lZD35 (ATCC 37565), pLXIN and pSIR (CLONTECH), pIRES-EGFP (CLONTECH). INVITROGEN corporation provides a wide variety of commercially available mammalian expression vector/systems which can be effectively used with the present invention. INVITROGEN, Carlsbad, CA. *See, also*, PHARMINGEN products, vectors and systems, San Diego, CA.

Baculoviral expression systems may also be used with the present invention to produce high yields of biologically active protein. Vectors such as the CLONETECH, BacPak™ Baculovirus expression system and protocols are preferred which are commercially available. CLONTECH, Palo Alto, CA. Miller, L.K., *et al.*, Curr. Op. Genet. Dev. 3:97 (1993); O'Reilly, D.R., *et al.*, *Baculovirus Expression Vectors: A Laboratory Manual*, 127. Vectors such as the INVITROGEN, MaxBac™ Baculovirus expression system, insect cells, and protocols are also preferred which are commercially available. INVITROGEN, Carlsbad, CA.

Example Host Cells

Host cells transformed with a nucleotide sequence which encodes a E3 ubiquitin ligase molecule of the present invention may be cultured under conditions suitable for the expression and recovery of the encoded protein from cell culture. Particularly preferred embodiments of the present invention are host cells transformed with a purified polynucleotide comprising a nucleic acid sequence encoding the polypeptide having the

sequence substantially as depicted in SEQ ID NO:3 or a biologically active fragment thereof. Cells of this type or preparations made from them may be used to screen for pharmacologically active modulators of the activity of the human E3 ubiquitin ligase. Modulators thus identified will be used for the treatment of disorders manifested by aberrant forms and/or abnormal levels of the native E3 ubiquitin protein ligase. Eukaryotic recombinant host cells are especially preferred as otherwise descibed herein or are well known to those skilled in the art. Examples include but are not limited to yeast, mammalian cells including but not limited to cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to Drosophila and silkworm derived cell lines. Cell lines derived from mammalian species which may be suitable and which are commercially available, include but are not limited to, L cells L-M(TK-) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616),BS-C-1 (ATCC CCL 26) and MRC-5 (ATCC CCL 171). The expression vector may be introduced into host cells expressing the ubiquitin ligase polypeptide *via* any one of a number of techniques including but not limited to transformation, transfection, lipofection, protoplast fusion, and electroporation. Commercially available kits applicable for use with the present invention for hererologous expression, including well-characterized vectors, transfection reagents and conditions, and cell culture materials are well-established and readily available. CLONTECH, Palo Alto, CA; INVITROGEN, Carlsbad, CA; PHARMINGEN, San Diego, CA; STRATAGENE, LaJolla, CA. The expression vector-containing cells are clonally propagated and individually analyzed to determine the level of the novel E3 ubiquitin protein ligase production. Identification of host cell clones which express the polypeptide may be performed by several means, including but not limited to immunological reactivity with antibodies described herein, and/or the presence of host cell-associated specific E3 ubiquitin protein ligase activity, and/or the ability to covalently cross-link specific substrate to the E3 ubiquitin protein ligase polypeptide with the bifunctional cross-linking reagent disuccinimidyl suberate or similar cross-linking reagents.

The ubiquitin protein ligase biomolecule of the present invention may also be expressed as a recombinant protein with one or more additional polypeptide domains added to facilitate protein purification. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals (Porath, J., Protein Exp. Purif., 3:263 (1992)), protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle WA). The inclusion of a cleavable linker sequences such as Factor XA or enterokinase (Invitrogen, San Diego CA) between the purification domain and the ubiquitin protein ligase coding region is useful to facilitate purification.

Systems such as the CLONTECH, TALON™ nondenaturing protein purification kit for purifying 6xHis-tagged proteins under native conditions and protocols are preferred which are commercially available. CLONTECH, Palo Alto, CA.

In addition, a host cell strain may be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation and acylation. Post-translational processing which cleaves a nascent form of the protein may also be important for correct insertion, folding and/or function. Different host cells such as CHO, HeLa, MDCK, 293, WI38, NIH-3T3, HEK293 etc., have specific cellular machinery and characteristic mechanisms for such post-translational activities and may be chosen to ensure the correct modification and processing of the introduced, foreign protein.

For long-term, high-yield production of the recombinant molecule, stable expression is preferred. For example, cell lines which stably express the novel E3 ubiquitin protein ligase polypeptide may be transformed using expression vectors which contain viral origins of replication or endogenous expression elements and a selectable marker gene. Following the introduction of the vector, cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells which

successfully express the introduced sequences. Resistant clumps of stably transformed cells can be proliferated using tissue culture techniques appropriate to the cell type.

The human E3 ubiquitin protein ligase and variations thereof described herein can be produced in the yeast *S.cerevisiae* following the insertion of the optimal cDNA cistron into expression vectors designed to direct the intracellular or extracellular expression of the heterologous protein. In the case of intracellular expression, vectors such as EmBLyex4 or the like are ligated to the beta subunit cistron. *See, e.g.*, Rinas, U., *et al.*, Biotechnology, 8:543 (1990); Horowitz, B., *et al.*, J. Biol. Chem., 265:4189 (1989). For extracellular expression, a ubiquitin protein ligase coding region, e.g., SEQ ID NO:2, is ligated into yeast expression vectors which may employ any of a series of well-characterized secretion signals. Levels of the expressed ubiquitin ligase molecule may be determined, for example, by means of the assays described herein.

A variety of protocols for detecting and measuring the expression of the human E3 ubiquitin protein ligase, using either polyclonal or monoclonal antibodies specific for the protein are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes may be employed. Well known competitive binding techniques may also be employed. See, e.g., Hampton, R., *et al.* (1990), *Serological Methods - a Laboratory Manual*, APS Press, St Paul Minn.; Maddox, D.E., *et al.*, J. Exp. Med. 158:1211.

Example Transformations

E coli transformations are generally carried out *via* electroporation. 400 ml cultures of strains DH5a or BL21(DE3) are grown in L-broth to an OD 600 of 0.5 and harvested at 2,000g. The cells are washed twice in ice-cold deionised water, resuspended in 1ml 10% glycerol and stored in aliquots at -70^{0}C. Ligation mixes are desalted using millipore V series membranes (0.0025mm pore). 40ml of cells are incubated with 1ul of ligation mix or plasmid DNA on ice for 10 minutes in 0.2cm electroporation cuvettes, then pulsed using a Gene Pulser apperatus (BioRad) at ^.5kVcm^{-1}, 25mF, 250 ½. Transformants are selected on L-agar supplimented with tertracyline at 10mg/ml or ampicillian at 100mg/ml.

Example Expression/purification

The novel human E3 ubiquitin protein ligase, e.g., SEQ ID NO:2, is expressed from a pET vector (e.g., 14b-16b *or* 28a-c(+) (NOVAGEN)) in BL21 cells, in such a way to produce a recombinant protein containing a 6-histidine tag immediately adjacent to the N-terminal methionine. The 6-His tag is used to aid purification of the recombinant protein as is passed through a nickel chelating column (NOVAGEN).

Over-expression of the Ubiquitin Ligase in cell-lines

Transient and/or stable eucaryotic transfectant cells comprised of the coding region(s) described herein are contemplated for high-level expression of the novel human E3 ubiquitin protein ligase as well as variations thereof.

Eucaryotic transfectants are preferred embodiments of the present invention for employment in studies for the identification molecules which modulate the human E3 ubiquitin protein ligase described herein *in vivo*. HEK cells, for example, may be employed.

Transient expression of coding regions for the human E3 ubiquitin protein ligase polypeptide can be achieved by straight transfection into mammalian cells, by standard techniques. Omari, K. *et al.*, J. Physiol., 499:369, (1997); Panyi, G. *et al.*, J. Gen. Physiol., 107(3):409 (1996). High level transient expression may be achieved using standard viral systems, e.g., Baculovirus, Adenovirus, or Vaccinia virus. Functionally expressed representatives resulting from these systems are typically 5-500K per cell. Kamb, A., Methods Enzymol. 207:423 (1992); Sun, T. *et al.*, Biochemistry, 33(33):9992 (1994); Spencer, R.H., *et al.*, J. Biol. Chem., 272:2389 (1997).

Stable transfection of heterologous cells using sequences which encode the novel E3 ubiquitin protein ligase described herein (SEQ ID NO:3) or pharmacologically active variations or fragments thereof can be generated using, for example, NIH-3t3, L929, COS, HEK, or CHO cells. *See, e.g.*, EMBO, 11(6):2033 (1992); Grissmer, *et al.*, Mol. Pharm., 45:1227 (1994).

A preferred vector for use with the present invention is pcDNA/Neo, which is commercially available from INVITROGEN, Carlsbad, CA.

Cells, NIH-3t3, for example, are grown to 50% confluency in 60mm plates (media and conditions are according to requirements of the particular cell line) and transfected with 5 ug of pure DNA comprising a coding region for the human E3 ubiquitin protein ligase, e.g. SEQ ID NO:2, in pCDNA/Neo using the Lipofection reagent, as described by the supplier (LIFE TECHNOLOGIES Gibco BRL, Bethesda, MD). After transfection, the cells are incubated at 37°C, conditions for 3 days in medium with 10% FCS. Cells are trypsinized seeded onto 100mm dishes, and then selected with 300ug/ml of G418 (Neomycin). Only cells that have stable integration of the heterologous coding region will grow in the presence of G418, which is confered by the Neomycin-resistance gene in the plasmid. Isolated clones are processed for 2-3 rounds of purification.

Example generation of Human 'Itchy' constructs

Human kidney cDNA was subjected to PCR using the following primers to isolate the full-length coding region of the human *itchy* E3 ligase (SEQ ID NO:3): upper: 5'-GTCTGACAGTGGATCACAAC-3' (SEQ ID NO:12); lower: 5'-CCATTCATGGTGCAAGTTCTC-3' (SEQ ID NO:13). PCR conditions were 94 ^{0}C 3 minutes; 31 cycles of 94 ^{0}C 1 minute, 58 ^{0}C 1 minute, 72 ^{0}C 2 minutes 30 seconds; 72 ^{0}C 3 minutes. The resulting 2616 bp product was cloned into the pCR2.1 TOPO TA cloning vector (Invitrogen, Carlsbad, CA) according to manufacturer's instructions except transformations were performed in ME DH5α cells. An N-terminal glutathione-S-transferase (GST)-itchy fusion construct was made by digesting the wild-type itchy TOPO vector with Eco RV and Spe I and cloning the purified insert into a Sma I-cut pGEX-5x-3 vector (Pharmacia, Piscataway, NJ) by blunt-end ligation (E3/pGEX-5x-3). An N-terminal green fluorescent protein (GFP) fusion construct for expression in mammalian cells was made by digesting the wild-type GST fusion construct with Bam HI and Not I and ligating the purified insert into the same sites in the pEGFP-C1 vector E3/pEGFP-C1 (Clontech, Palo Alto, CA). *Expression of gst fusion constructs*; *see* Example VI. Each of the anti-SEQ ID NO:3 peptide antibodies described herein (*infra*) were demonstrated via wetern blot to react strongly with the SEQ ID NO:3 fusion product.

E1

All ubiquitin-activating (E1) proteins and genes corresponding thereto are contemplated for use in biological assays as well as drug screen assays described herein. SEQ ID NO:10 (FIG.12), for example, as an embodiment for use in the methods described and contemplated herein, is the 3177 base translated structural coding region of the nucleic acid sequence which encodes the previously described 1058 amino acid residue human E1 ubiquitin activating enzyme (Uba1). Ayusawa, D., *et al.*, Cell Struct. Funct., 17:113 (1992). *See, also*, Jentsch, S., *et al., Genetic Analysis of the Ubiquitin System, Biochim. Biophys. Acta*, 1089:127 (1991); McGrath, J.P., *et al., UBA1 - An Essential Yeast Gene Encoding Ubiquitin-Activating Enzyme*, EMBO 10: 227 (1991); *Immunofluorescent Localization of the Ubiquitin-Activating Enzyme, E1, to the Nucleus and Cytoskeleton*, Am. J. Physiol, 264:C9;. Cook, J.C., *et al., Ubiquitin-Activating Enzyme in Cultured Cells*, PNAS, 92:3454 (1995); Nagai, Y., *et al., Ubiquitin-Activating Enzyme, E1, is Phosphorylated in Mammalian Cells by the Protein Kinase Cdc2*, J. Cell Sci., 108:2145 (1995).

E2

Similarly, all ubiquitin-conjugating enzymes (E2) proteins and genes corresponding thereto are contemplated for use in biological assays as well as drug screen assays described herein. SEQ ID NO:11 (FIG.13), for example, as an embodiment for use in the methods described and contemplated herein, is the 444 base translated structural coding region of the nucleic acid sequence which encodes the previously described 147 amino acid residue E2 ubiquitin conjugating enzyme E217k (ub10a). Wing S.S., *et al.*, Biochem. J., 305:125 (1995) [The E2 human version (Ubc2) is preferred as described by Koken, M., *et al.*, PNAS, 88:8865 (1991)]. Other embodiments of E2 ubiquitin conjugating enzymes for use in methods of the present invention include, but are not limited to: Ubc2/Rad6 (Koken, M., *et al.*, PNAS, 88:8865 (1991) *Human E2*), Ubc3/Cdc34 (Plon, *et al.*, PNAS, 90:10484 (1993)), Ubc4/Ubc5B (Jensen, *et al.*, J. Biol. Chem., 270:30408 (1995) & Rolfe, *et al.*, PNAS, 92:3264 (1995)), Ubc5/Ubc5A (Jensen, *et al.*, J. Biol. Chem., 270:30408 (1995) & Schneffer, et al., PNAS, 91:8797 (1994)), Ubc5C (Jensen, *et al.*, J. Biol. Chem., 270:30408 (1995)), Ubc6 (Nuber, et al., J Biol Chem 271:2795 (1996)), Ubc7 (Nuber, *et*

al., J Biol Chem 271:2795 (1996) & Robinson, *et al.*, Mammal Genome, 6:725 (1995)), Ubc8 (Kaiser, *et al.*, J Biol Chem, 269:8797 (1994)), Ubc9 (Kovalenko, *et al.*, PNAS, 93:2958 (1996)), Watanabe, *et al.*, Cytogen Cell Gen ., 72:86 (1996), Ubc-epi (Liu, *et al.*, *cDNA Cloning of a Novel Human Ubiquitin Carrier Protein*, J. Biol. Chem., 267:15829 (1992)), and Ubc-bendless: GENBANK Accession Number D83004. *See, generally*, Rolfe, *et al.*, *The Ubiquitin-Mediated Proteolytic Pathway as a Therapeutic Area, J. Mol. Med.*, 75:5 (1997)). *See, also*, Baboshina, O.V., *et al.*, *Novel Multiubiquitin Chain Linkages Catalysed by the Conjugating Enzymes E2(EPF) and RAD6 are recognized by the 26-S Proteasome Subunit*, J. Biol .Chem., 271:2823 (1996); Dohmen, R.J., *et al.*, *The N-End Rule Is Mediated by the Ubc2(Rad6) Ubiquitin-Conjugating Enzyme*, PNAS, 88:7351 (1991); Seufert, W., *et al.*, *Ubiquitin-Conjugating Enzymes Ubc4 and Ubc5 Mediate Selective Degradation of Short-Lived and Abnormal Proteins*, EMBO, 9:543 (1990); Cook, W.J., *et al.*, *3-Dimensional Structure of a Ubiquitin-Conjugating Enzyme (E2)*, J. Biol. Chem., 267:15116 (1992); Bartel, B., *et al.*, *The Recognition Component of the N-End Rule Pathway*, EMBO, 9:3179 (1990).

Ubiquitin

Ubiquitin is available, labeled and unlabeled, from a variety of well-known commercial suppliers. SEQ ID NO:7 is the 156 amino acid precursor peptide to the mature 76 amino acid residue sequence of human ubiquitin (FIG.9) (Lund P.K., *et al.*, J. Biol. Chem., 260:7609 (1985)). Figure 10 displays SEQ ID NO:8 which is the mature 76 amino acid residue sequence of native human ubiquitin (positions 1-76 of SEQ ID NO:7). SEQ ID NO:9 (FIG.11) is the 471 base translated structural coding region of the cDNA nucleic acid sequence which encodes the156 amino acid precursor peptide (SEQ ID NO:7) to the mature 76 amino acid residue sequence of native human ubiquitin (positions 1-76 of SEQ ID NO:7) (Lund P.K., *et al.*, J. Biol. Chem., 260:7609 (1985)). The human E3 ubiquitin protein ligase (SEQ ID NO:3) ubiquitinates specific intracellular biological molecules *in vivo* including the likes of intracellular messenger biological molecules, receptors, ligands, signal transduction molecules, transcriptional activators, cytokines, kinases, and phosphorylases, especially which mediate physiological conditions such as inflammation, autoimmune disease, neurological disease, apoptosis, endothelial cell physiology (e.g.,

proliferation, differentiation), peripheral vascular disease, angiogenesis, cancer, anemia, hematopoietic disorders, cachexia, leukemia, pulmonary disorders, arthritis, diabetes, and viral infection to effect selective destruction and swift regulation of cellular physiology. Any potential substrate may be used in biological assays as well as drug screen assays described herein including, but not limited to, substrates referred to in references cited herein or which are otherwise known or identified in the art of human pathophysiology.

General Biological Assays

Methods of identifying compounds that modulate the biological activity of a human E3 ubiquitin protein ligase, are contemplated and provided herein and in the EXAMPLES which comprise combining a candidate compound modulator of human E3 ubiquitin protein ligase biological activity with a human E3 ubiquitin protein ligase polypeptide having the sequence substantially as depicted in SEQ ID NO:3, and measuring an effect of the candidate compound modulator on the biological activity.

General Pharmacological Assays

The human E3 ubiquitin protein ligase described herein may be assayed for its ability to modulate protein degradation or selective proteolysis and/or otherwise modulate conditions associated with aberrant ubiquitin dependent proteolysis in intracellular physiology (disorders manifested by aberrant forms and/or abnormal levels of the native E3 ubiquitin protein ligase). Methods of identifying compounds that modulate the pharmacological activity of a human E3 ubiquitin protein ligase, comprise combining a candidate compound modulator of human E3 ubiquitin protein ligase pharmacological activity with a host-cell expressing a human E3 ubiquitin protein ligase polypeptide having the sequence substantially as depicted in SEQ ID NO:3, and measuring an effect of the candidate compound modulator on the pharmacological activity.

Scintillation Proximity Assay

Scintillation Proximity Assay (SPA) technology is developed which allows the rapid and sensitive assay of a wide variety of molecular interactions in a homogeneous system. AMERSHAM, Bucks, UK. The decay of a radioactive atom releases sub-atomic particle radiation. The distance these particles travel through the medium in which they are released is dependent upon the energy of the particle. In the scintillation proximity assay

scintillant is incorporated into small fluoromicrospheres. These microspheres or 'beads' are derivatized in such a way as to bind specific molecules. If a radioactive molecule is bound to the bead, the radiation is in close enough proximity to stimulate the scintillant in order to emit light (unbound isotopes are too distant). The technique of SPA simplifies the process of assay design by removing the necessity to separate bound from free ligand, allowing assays to be performed and counted in one tube or in 96-well microplates. Moreover, assay speed is increased, and the need for filters, solvents, vials and scintillation reagents is eliminated. SPA is employed in screening assays as diverse as protein:protein, protein:DNA and cell adhesion molecule interactions. SPA represents a major drug screening technology, which has already been used successfully to identify a large number of candidate therapeutic compounds against a multitude of targets. See, EXAMPLE III. Figure 8 shows a schematic representation of example Scintillation Proximity Assays (SPA), as well as RIA and ELISA Assays.

Human E3 ubiquitin protein ligase may be therefore assayed for inherent pharmacological properties which may be useful to exploit for therapeutic purposes, i.e., administration *via* gene therapy or otherwise, *in vivo*, to control the selective elimination of intracellular biomolecules and hence regulate physiology.

Ubiquination Assays

Ubiquination reactions were performed using a protocol based on that used by Hatakeyama, *et al.* J. Biol. Chem., 272:15085 (1997). *See*, Example VII; FIG.14.

Various Screening Assays

The present invention is also directed to methods for screening for compounds which modulate the biological and/or pharmacological activity of the human E3 ubiquitin protein ligase. Compounds which modulate these activities may be DNA, RNA, peptides, proteins, or non-proteinaceous organic molecules. Compounds may modulate the activity by increasing or attenuating the expression of DNA or RNA which encode the biomolecule, or may antagonize or agonize the activity of the human E3 ubiquitin protein ligase itself. Compounds that modulate the expression of DNA or RNA encoding the subunit or the function of the polypeptide may be detected by a variety of assays. The assay may be a simple "yes/no" assay to determine whether there is a change in expression or function. The

assay may be made quantitative by comparing the expression or function of a test sample with the levels of expression or function in a standard sample.

The human E3 ubiquitin protein ligase described herein, its immunogenic fragments or oligopeptides can be used for screening therapeutic compounds in any of a variety of drug screening techniques. The fragment employed in such a test may be free in solution, affixed to a solid support, borne on a cell surface, or located intracellularly. The abolition of activity or the formation of binding complexes, between the E3 ubiquitin ligase biomolecule and the agent being tested, may be measured. Accordingly, the present invention provides a method for screening a plurality of compounds for specific binding affinity with the human E3 ubiquitin protein ligase polypeptide or a fragment thereof, comprising providing a plurality of compounds; combining a polypeptide of the present invention or a fragment thereof with each of a plurality of compounds for a time sufficient to allow binding under suitable conditions; and detecting binding of the subunit, or fragment thereof, to each of the plurality of compounds, thereby identifying the compounds which specifically bind the human E3 ubiquitin protein ligase. Compounds that modulate the biological activity of human E3 ubiquitin protein ligase identified in this manner are especially preferred embodiments of the invention. A further embodiment of the present invention is a method of treatment of a patient in need of such treatment for a condition which is mediated by the biological activity of human E3 ubiquitin protein ligase comprising administration of a modulating compound which was identified by means of a method described herein. A further embodiment of the present invention is a method of treatment of a patient in need of such treatment for a condition which is mediated by a pharmacological activity of human E3 ubiquitin protein ligase comprising administration of a modulating compound which was identified by means of a method described herein.

In order to purify an E3 protein ligase polypeptide to measure a binding activity, the source may be a whole cell lysate, prepared by one to three freeze-thaw cycles in the presence of standard protease inhibitors. The protein ligase may be partially or completely purified by standard protein purification methods. Human E3 ubiquitin protein ligase polypeptides described herein may be purified by affinity chromatography using antibodies described herein or by ligands specific for an epitope tag engineered into the recombinant molecule

moreover described herein. The preparation may then be assayed for binding activity as described.

Purified polypeptides comprising the amino acid sequence substantially as depicted in SEQ ID NO:3 are especially preferred embodiments of the present invention.

Compounds and Methods

Compounds which are identified generally according to methods described, referenced, and contemplated herein that modulate the biological and/or pharmacological activity of human E3 ubiquitin protein ligase (SEQ ID NO:3) are especially preferred embodiments of the present invention. Therefore, as an inherent corollary, a method of the present invention is treatment of a patient in need of such treatment for a condition which is mediated by the biological and/or pharmacological activity of a human E3 ubiquitin protein ligase, comprising administration of a compound that modulates the biological and/or pharmacological activity of a human E3 ubiquitin protein ligase identified by a method described herein.

A further method of the present invention is treatment of a patient in need of such treatment for a condition which is mediated by the biological and/or pharmacological activity of a human E3 ubiquitin protein ligase, comprising administration of the E3 ubiquitin protein ligase substantially as depicted in SEQ ID NO:3 or a pharmacologically active fragment thereof. Therapeutic methods of the present invention also include treatment of a patient in need of such treatment for a condition which is mediated by the biological and/or pharmacological activity of a human E3 ubiquitin protein ligase, comprising administration of a nucleic acid substantially as depicted in SEQ ID NO:1 or a biologically-effective fragment thereof. Therapeutic methods of the present invention furthermore include treatment of a patient in need of such treatment for a condition which is mediated by the biological activity of a human E3 ubiquitin protein ligase, comprising administration of an antisense molecule comprising the complement of the sequence substantially as depicted in SEQ ID NO:2 or a biologically-effective fragment thereof (further discussed *infra*).

Yeast 2-Hybrid System

In another embodiment of the invention, a nuleic acid sequence which encodes a human E3 ubiquitin protein ligase molecule substantially as depicted in SEQ ID NO:3 or a

biologically and/or pharmacologically active fragment thereof may be ligated to a heterologous sequence to encode a fusion protein, for example, to encode a chimeric human E3 ubiquitin ligase molecule as described herein for expression in hererologous host cells for screening molecules for an ability to modulate human E3 ubiquitin protein ligase biological and/or pharmacological activity, i.e., *via* binding, association or otherwise. Chimeric constructs may also be used to express a 'bait', according to methods well known using a yeast two-hybrid system, to identify accessory native peptides that may be associated with the human E3 ubiquitin protein ligase described herein. Fields, S., *et al.*, Trends Genet., 10:286 (1994); Allen, J.B., *et al.*, TIBS, 20:511 (1995). A yeast two-hybrid system has been described wherein protein:protein interactions can be detected using a yeast-based genetic assay via reconstitution of transcriptional activators. Fields, S., Song, O., Nature 340:245 (1989). The two-hybrid system used the ability of a pair of interacting proteins to bring a transcription activation domain into close proximity with a DNA-binding site that regulates the expression of an adjacent reporter gene. Commercially available systems such as the CLONTECH, Matchmaker™ systems and protocols may be used with the present invention. CLONTECH, Palo Alto, CA. *See also*, Mendelsohn, A.R., Brent, R., Curr. Op. Biotech., 5:482 (1994); Phizicky. E.M., Fields, S., Microbiological Rev., 59(1):94 (1995); Yang, M., et al., Nucleic Acids Res., 23(7):1152 (1995); Fields, S., Sternglanz, R., TIG, 10(8):286 (1994); and US Patents 5,283,173, *System to Detect Protein-Protein Interactions*, and 5,468,614, which are incorporated herein by reference.

Modified screening systems, for instance, can be practiced either with a positive readout or with a negative readout such as that in the recently developed versions of "Reverse Y2H" approach. *See, e.g.*, Vidal M, Braun P, Chen E, Boeke JD, Harlow E (1996) *Genetic characterization of a mammalian protein-protein interaction domain by using a yeast reverse two-hybrid system*, Proc Natl Acad Sci U S A 17;93(19):10321-10326; Vidal M, Brachmann RK, Fattaey A, Harlow E, Boeke JD (1996) *Reverse two-hybrid and one-hybrid systems to detect dissociation of protein-protein and DNA-protein interactions*. Proc Natl Acad Sci U S A 17;93(19):10315-10320; White MA (1996) *The yeast two-hybrid system: forward and reverse*, Proc Natl Acad Sci U S A 17;93(19):10001-10003; Leanna

CA, Hannink M (1996), *The reverse two-hybrid system: a genetic scheme for selection against specific protein/protein interactions*, Nucleic Acids Res 1;24(17):3341-3347.

Antibodies

Example peptides and anti-peptide antibodies were made by under contract by Genosys Inc., Woodlands, Tx. The two peptide sequences used to generate polyclonal antibodies to human itchy E3 ligase (SEQ ID NO:3) were amino acid positions 41-54 and positions 153-167 of SEQ ID NO:3. Peptides were conjugated to KLH (2-3 mg). New Zealand White rabbits were independently immunized each with 200 mg of one conjugated peptide in Complete Freund's adjuvant for the first immunization and with 100 mg of tha same conjugated peptide in Incomplete Freund's Adjuvant for subsequent immunizations. Anti-peptide antibodies was affinity-purified by elution through a column containing Affi-gel 10 matrix (BioRad, Hercules, CA) to which each respective free peptide had been conjugated. Monospecific antibodies to the human biomolecule of the present invention (SEQ ID NO:3) are purified from mammalian antisera containing antibodies reactive against the polypeptide or are prepared as monoclonal antibodies reactive with the human E3 ubiquitin protein ligase using the technique of Kohler and Milstein, Nature, 256:495 (1975). Mono-specific antibody as used herein is defined as a single antibody species or multiple antibody species with homogenous binding characteristics for the novel human E3 ubiquitin protein ligase. Homogenous binding as used herein refers to the ability of the antibody species to bind to a specific antigen or epitope. Human E3 ubiquitin protein ligase specific antibodies are raised by immunizing animals such as mice, rats, guinea pigs, rabbits, goats, horses and the like, with rabbits being preferred, with an appropriate concentration of the human E3 ubiquitin protein ligase either with or without an immune adjuvant.

Preimmune serum is collected prior to the first immunization. Each animal receives between about 0.1 mg and about 1000 mg of human E3 ubiquitin protein ligase polypeptide associated with an acceptable immune adjuvant. Such acceptable adjuvants include, but are not limited to, Freund's complete, Freund's incomplete, alum-precipitate, water in oil emulsion containing Corynebacterium parvum and tRNA. The initial immunization consists of a human E3 ubiquitin protein ligase polypeptide in, preferably, Freund's complete adjuvant at multiple sites either subcutaneously (SC), intraperitoneally (IP) or

both. Each animal is bled at regular intervals, preferably weekly, to determine antibody titer. The animals may or may not receive booster injections following the initial immunization. Those animals receiving booster injections are generally given an equal amount of the antigen in Freund's incomplete adjuvant by the same route. Booster injections are given at about three week intervals until maximal titers are obtained. At about 7 days after each booster immunization or about weekly after a single immunization, the animals are bled, the serum collected, and aliquots are stored at about - 20° C. Monoclonal antibodies (mAb) reactive with the human E3 ubiquitin protein ligase polypeptide are prepared by immunizing inbred mice, preferably Balb/c, with a human E3 ubiquitin protein ligase polypeptide. The mice are immunized by the IP or SC route with about 0.1 mg to about 10 mg, preferably about 1 mg, of the novel protein ligase polypeptide in about 0.5 ml buffer or saline incorporated in an equal volume of an acceptable adjuvant, as discussed above. Freund's complete adjuvant is preferred. The mice receive an initial immunization on day 0 and are rested for about 3 to about 30 weeks. Immunized mice are given one or more booster immunizations of about 0.1 to about 10 mg of human E3 ubiquitin protein ligase polypeptide in a buffer solution such as phosphate buffered saline by the intravenous (IV) route. Lymphocytes, from antibody positive mice, preferably splenic lymphocytes, are obtained by removing spleens from immunized mice by standard procedures known in the art. Hybridoma cells are produced by mixing the splenic lymphocytes with an appropriate fusion partner, preferably myeloma cells, under conditions which will allow the formation of stable hybridomas. Fusion partners may include, but are not limited to: mouse myelomas P3/NS1/Ag 4-1; MPC-11; S-194 and Sp 2/0, with Sp 2/0 being preferred. The antibody producing cells and myeloma cells are fused in polyethylene glycol, about 1000 molecular weight, at concentrations from about 30% to about 50%. Fused hybridoma cells are selected by growth in hypoxanthine, thymidine and aminopterin supplemented Dulbecco's Modified Eagles Medium (DMEM) by procedures known in the art. Supernatant fluids are collected from growth positive wells on about days 14, 18, and 21 and are screened for antibody production by an immunoassay such as solid phase immunoradioassay (SPIRA) using a human E3 ubiquitin protein ligase polypeptide as the antigen. The culture fluids are also tested in the

Ouchterlony precipitation assay to determine the isotype of the mAb. Hybridoma cells from antibody positive wells are cloned by a technique such as the soft agar technique of MacPherson, Soft Agar Techniques, in Tissue Culture Methods and Applications, Kruse and Paterson, Eds., Academic Press, (1973).

Monoclonal antibodies are produced *in vivo* by injection of pristane primed Balb/c mice, approximately 0.5 ml per mouse, with about 2×10^6 to about 6×10^6 hybridoma cells about 4 days after priming. Ascites fluid is collected at approximately 8-12 days after cell transfer and the monoclonal antibodies are purified by techniques known in the art.

In vitro production of the anti- human E3 ubiquitin protein ligase polypeptide mAb is carried out by growing the hydridoma in DMEM containing about 2% fetal calf serum to obtain sufficient quantities of the specific mAb. The mAb are purified by techniques well known in the art.

Diagnostic Assays

Antibody titers of ascites or hybridoma culture fluids are determined by various serological or immunological assays which include, but are not limited to, precipitation, passive agglutination, enzyme-linked immunosorbent antibody (ELISA) technique and radioimmunoassay (RIA) techniques. Similar diagnostic assays are used to detect the presence of the human E3 ubiquitin protein ligase in body fluids or tissue and cell extracts. Diagnostic assays using the human E3 ubiquitin protein ligase polypeptide specific antibodies are useful for the diagnosis of conditions manifested by aberrant forms and/or abnormal levels and/or tissue distribution of the native E3 ubiquitin protein ligase. Diagnostic assays for the human ubiquitin ligase biomolecule of this invention include methods utilizing the antibody and a label to detect the human E3 ubiquitin protein ligase polypeptide in human body fluids, cells, tissues or sections or extracts of such tissues. The polypeptides and antibodies of the present invention may be used with or without modification. Frequently, the polypeptides and antibodies will be labeled by joining them, either covalently or noncovalently, with a reporter molecule, a myriad of which are well-known to those skilled in the art.

A variety of protocols for measuring the human E3 ubiquitin protein ligase polypeptide, using either polyclonal or monoclonal antibodies specific for the respective protein are

known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on the human E3 ubiquitin protein ligase is preferred, but a competitive binding assay may be employed. These assays are described, among other places, in Maddox, D.E. *et al.*, J. Exp. Med. 158:1211 (1983); Sites, D.P., *et al.*, *Basic and Clinical Immunology*, Ch.22, 4th Ed., Lange Medical Publications, Los Altos, CA (1982); U.S. Patents No. 3,654,090, No. 3,850,752; and No. 4,016,043.

In order to provide a basis for the diagnosis of disease, normal or standard values for the human E3 ubiquitin protein ligase polypeptide expression must be established. This is accomplished by combining body fluids or cell extracts taken from normal subjects, either animal or human, with antibody to the human ubiquitin ligase biomolecule under conditions suitable for complex formation which are well known in the art. The amount of standard complex formation may be quantified by comparing it with a dilution series of positive controls where a known amount of antibody is combined with known concentrations of purified E3 ubiquitin protein ligase polypeptide. Standard values obtained from normal samples may be compared with values obtained from samples from subjects potentially affected by a disorder or disease related to expression of the human E3 ubiquitin protein ligase. Deviation between standard and subject values establishes the presence of the disease state.

Kits which contain human E3 ubiquitin protein ligase nucleic acid coding region(s), antibodies to a polypeptide, or intact biomolecule may be prepared. Such kits are used to detect sample nucleic acids which hybridize to the human E3 ubiquitin protein ligase nucleic acid coding region(s) contained therein, or to detect the presence of the intact biomolecule or peptide fragments in a sample. Such characterization is useful for a variety of purposes including, but not limited to, diagnosis, forensic analyses and epidemiological studies.

The DNA molecules, RNA molecules, recombinant protein and antibodies of the present invention may be used to screen and measure levels of the human E3 ubiquitin protein ligase DNA, RNA or protein. The recombinant proteins, DNA molecules, RNA molecules

and antibodies lend themselves to the formulation of kits suitable for the detection and typing of the novel human E3 ubiquitin protein ligase biomolecule. Such a kit would comprise a compartmentalized carrier suitable to hold in close confinement at least one container. The carrier would further comprise reagents such as recombinant ubiquitin protein ligase or anti- ubiquitin protein ligase antibodies suitable for detecting the novel human E3 ubiquitin protein ligase biomolecule. The carrier may also contain a means for detection such as labeled antigen or enzyme substrates or the like.

Polynucleotide sequences which encode the human E3 ubiquitin protein ligase may be used for the diagnosis of conditions or diseases with which the expression of the human biomolecule is associated. For example, polynucleotide sequences encoding the human E3 ubiquitin protein ligase may be used in hybridization or PCR assays of fluids or tissues from biopsies to detect expression of the biomolecule. The form of such qualitative or quantitative methods may include Southern or Northern analysis, dot blot or other membrane-based technologies; PCR technologies; dip stick, pin, chip and ELISA technologies. All of these techniques are well known in the art and are the basis of many commercially available diagnostic kits. Such assays may also be used to evaluate the efficacy of a particular therapeutic treatment regimen in animal studies, in clinical trials, or in monitoring the treatment of an individual patient. Once disease is established, a therapeutic agent may be administered and a treatment profile generated. Such assays may be repeated on a regular basis to evaluate whether the values in the profile progress toward or return to the normal or standard pattern. Successive treatment profiles may be used to show the efficacy of treatment over a period of several days or several months.

Polynucleotide sequences which encode the human E3 ubiquitin protein ligase may also be employed in analyses to map chromosomal locations, e.g., screening for functional association with disease markers. Moreover the sequences described herein are contemplated for use to identify human sequence polymorphisms and possible association with disease as well as analyses to select optimal sequence from among possible polymorphic sequences for the design of compounds to modulate the biological activity and therefore regulate physiological disorders. Furthermore the sequences are

contemplated as screening tools for use in the identification of appropriate human subjects and patients for therapeutic clinical trials.

Purification *via* Affinity Columns

It is readily apparent to those skilled in the art that methods for producing antibodies may be utilized to produce antibodies specific for human E3 ubiquitin protein ligase polypeptide fragments, or the full-length nascent human polypeptide. Specifically, it is readily apparent to those skilled in the art that antibodies may be generated which are specific for the fully functional biomolecule or fragments thereof.

Human E3 ubiquitin protein ligase antibody affinity columns are made by adding the antibodies to Affigel-10 (Biorad), a gel support which is activated with N hydroxysuccinimide esters such that the antibodies form covalent linkages with the agarose gel bead support. The antibodies are then coupled to the gel via amide bonds with the spacer arm. The remaining activated esters are then quenched with 1M ethanolamine HCl (pH 8). The column is washed with water followed by 0.23M glycine HCl (pH 2.6) to remove any non-conjugated antibody or extraneous protein. The column is then equilibrated in phosphate buffered saline (pH 7.3) with appropriate detergent and the cell culture supernatants or cell extracts, for example, containing human E3 ubiquitin protein ligase polypeptide made using appropriate membrane solubilizing detergents are slowly passed through the column. The column is then washed with phosphate buffered saline/detergent until the optical density falls to background, then the protein is eluted with 0.23M glycine-HCl (pH 2.6)/detergent. The purified subunit polypeptide is then dialyzed against phosphate buffered saline/detergent.

Recombinant E3 ubiquitin protein ligase molecules can be separated from other cellular proteins by use of an immunoaffinity column made with monoclonal or polyclonal antibodies specific for the full length nascent human protein, e.g., SEQ ID NO:3, or polypeptide fragments of the biomolecule.

Human E3 ubiquitin protein ligase as described herein may be used to affinity purify biological effectors from native biological materials, e.g. disease tissue. Affinity chromatography techniques are well known to those skilled in the art. The novel polypeptide described herein, e.g., SEQ ID NO:3, or an effective fragment thereof, is fixed

to a solid matrix, e.g. CNBr activated Sepharose according to the protocol of the supplier (Pharmacia, Piscataway, NJ), and a homogenized/buffered cellular solution containing a potential molecule of interest is passed through the column. After washing, the column retains only the biological effector which is subsequently eluted, e.g., using 0.5M acetic acid or a NaCl gradient.

Example Dominant Negative Mutant

To prove that the active site cysteine in the human itchy gene is essential for ubiquitination, bacterially expressed protein from the dominant negative construct (C820A, described herein) was used in ubiquitination assays. The results (Example VIII; FIG.15) demonstrate that the dominant negative construct (C820A) has no enzymatic activity (ubiquitination) compared to the wild type control. See Example VIII; FIG.15. Ubiquitination substrate (DH5a bacterial Lysates) · 12% SDS-PAGE · Primary Ab (anti-ubiquitin) · Secondary Ab (Anti-rabbit Ig) · Western blot developed using ECL system (Amersham, Bucks, UK).

Antisense Molecules

To enable methods of down-regulating expression of the human E3 ubiquitin protein ligase of the present invention in mammalian cells, an example antisense expression construct containing the complement DNA sequence to the sequence substantially as depicted in SEQ ID NO:2 can be readily constructed for instance using the pREP10 vector (Invitrogen Corporation). Transcripts are expected to inhibit translation of the wild-type E3 ubiquitin protein ligase mRNA in cells transfected with this type construct. Antisense transcripts are effective for inhibiting translation of the native gene transcript, and capable of inducing the effects (e.g., regulation of physiological disorders) herein described. Translation is most effectively inhibited by blocking the mRNA at a site at or near the initiation codon. Thus, oligonucleotides complementary to the corresponding 5' -terminal region of the human E3 ubiquitin protein ligase mRNA transcript (SEQ ID NO:2) are preferred. Secondary or tertiary structure which might interfere with hybridization is minimal in this region. Moreover, sequences that are too distant in the 3' direction from the initiation site can be less effective in hybridizing the mRNA transcripts because of a "read-through" phenomenon whereby the ribosome appears to unravel the antisense/sense duplex to permit translation of the message. Oligonucleotides which are complementary to and hybridizable

with any portion of the human E3 ubiquitin protein ligase mRNA are contemplated for therapeutic use.

U.S. Patent No. 5,639,595, *Identification of Novel Drugs and Reagents*, issued Jun. 17, 1997, wherein methods of identifying oligonucleotide sequences that display *in vivo* activity are thoroughly described, is herein incorporated by reference. Expression vectors containing random oligonucleotide sequences derived from previously known polynucleotides, e.g., SEQ ID NO:1, are transformed into cells. The cells are then assayed for a phenotype resulting from the desired activity of the oligonucleotide. Once cells with the desired phenotype have been identified, the sequence of the oligonucleotide having the desired activity can be identified. Identification may be accomplished by recovering the vector or by polymerase chain reaction (PCR) amplification and sequencing the region containing the inserted nucleic acid material.

Nucleotide sequences that are complementary to the novel E3 ubiquitin protein ligase polypeptide encoding polynucleotide sequence can be synthesized for antisense therapy. These antisense molecules may be DNA, stable derivatives of DNA such as phosphorothioates or methylphosphonates, RNA, stable derivatives of RNA such as 2'-O-alkylRNA, or other oligonucleotide mimetics. U.S. Patent No. 5,652,355, *Hybrid Oligonucleotide Phosphorothioates*, issued July 29, 1997, and U.S. Patent No. 5,652,356, *Inverted Chimeric and Hybrid Oligonucleotides*, issued July 29, 1997, which describe the synthesis and effect of physiologically-stable antisense molecules, are incorporated by reference. Human E3 ubiquitin protein ligase antisense molecules may be introduced into cells by microinjection, liposome encapsulation or by expression from vectors harboring the antisense sequence. Antisense therapy may be particularly useful for the treatment of diseases where it is beneficial to modulate the biological activity and/or pharmacological activity of the human E3 ubiquitin protein ligase described herein.

Gene Therapy

The human E3 ubiquitin protein ligase polypeptide and variations thereof contemplated herein may administered to a subject *via* gene therapy. A polypeptide of the present invention may be delivered to the cells of target organs, e.g., hematopoietic cells, in this manner. Conversely, human E3 ubiquitin protein ligase polypeptide *antisense* gene therapy

may be used to modulate the expression of the polypeptide in the same cells of target organs and hence regulate biological and/or pharmacological activity. The human E3 ubiquitin protein ligase coding region can be ligated into viral vectors which mediate transfer of the trans-activator polypeptide nucleic acid by infection of recipient host cells. Suitable viral vectors include retrovirus, adenovirus, adeno-associated virus, herpes virus, vaccinia virus, polio virus and the like. *See, e.g.*, U.S. Patent No. 5,624,820, *Episomal Expression Vector for Human Gene Therapy*, issued April 29, 1997.

Nucleic acid coding regions of the present invention are incorporated into effective eukaryotic expression vectors, which are directly administered or introduced into somatic cells for gene therapy (a nucleic acid fragment comprising a coding region, preferably mRNA transcripts, may also be administered directly or introduced into somatic cells). *See, e.g.*, U.S. Patent No. 5,589,466, issued Dec. 31, 1996. Such nucleic acids and vectors may remain episomal or may be incorporated into the host chromosomal DNA as a provirus or portion thereof that includes the gene fusion and appropriate eukaryotic transcription and translation signals, i.e, an effectively positioned RNA polymerase promoter 5' to the transcriptional start site and ATG translation initiation codon of the gene fusion as well as termination codon(s) and transcript polyadenylation signals effectively positioned 3' to the coding region. Alternatively, the human E3 ubiquitin protein ligase DNA can be transferred into cells for gene therapy by non-viral techniques including receptor-mediated targeted DNA transfer using ligand-DNA conjugates or adenovirus-ligand-DNA conjugates, lipofection membrane fusion or direct microinjection. These procedures and variations thereof are suitable for *ex vivo*, as well as *in vivo* human gene therapy according to established methods in this art.

PCR Diagnostics

The nucleic acid sequence, oligonucleotides, fragments, portions or antisense molecules thereof, may be used in diagnostic assays of body fluids or biopsied tissues to detect the expression level of the human E3 ubiquitin protein ligase. For example, sequences designed from the cDNA sequence SEQ ID NO:1 or sequences comprised in SEQ ID NO:2 can be used to detect the presence of the mRNA transcripts in a patient or to monitor the modulation of transcripts during treatment.

Figure 7 displays PCR primers, for example, SEQ ID NO:5 and SEQ ID NO:6, which are used to amplify the 2559 bp coding region (SEQ ID NO:2) of the novel human E3 ubiquitin protein ligase from human tissue.

One method for amplification of target nucleic acids, or for later analysis by hybridization assays, is known as the polymerase chain reaction ("PCR") or PCR technique. The PCR technique can be applied to detect sequences of the invention in suspected samples using oligonucleotide primers spaced apart from each other and based on the genetic sequence, e.g., SEQ ID NO:1, set forth herein. The primers are complementary to opposite strands of a double stranded DNA molecule and are typically separated by from about 50 to 450 nucleotides or more (usually not more than 2000 nucleotides). This method entails preparing the specific oligonucleotide primers followed by repeated cycles of target DNA denaturation, primer binding, and extension with a DNA polymerase to obtain DNA fragments of the expected length based on the primer spacing. One example embodiment of the present invention is a diagnostic composition for the identification of a polynucleotide sequence comprising the sequence substantially as depicted in SEQ ID NO:2 comprising the PCR primers substantially as depicted in SEQ ID NO:5 and SEQ ID NO:6 (FIG.7). The degree of amplification of a target sequence is controlled by the number of cycles that are performed and is theoretically calculated by the simple formula 2n where n is the number of cycles. *See, e.g.*, Perkin Elmer, *PCR Bibliography*, Roche Molecular Systems, Branchburg, New Jersey; CLONTECH products, Palo Alto, CA; U.S. Patent No. 5,629,158, *Solid Phase Diagnosis of Medical Conditions*, issued May 13, 1997.

Compositions

Pharmaceutically useful compositions comprising sequences pertaing to the human E3 ubiquitin protein ligase, DNA, RNA, antisense sequences, or variants and analogs which have biological activity or otherwise compounds which modulate cell physiology identified by methods described herein, may be formulated according to known methods such as by the admixture of a pharmaceutically acceptable carrier. Examples of such carriers and methods of formulation may be found in *Remington's Pharmaceutical Sciences* (Maack Publishing Co, Easton, PA). To form a pharmaceutically acceptable composition suitable

for effective administration, such compositions will contain an effective amount of the protein, DNA, RNA, or compound modulator.

Therapeutic or diagnostic compositions of the invention are administered to an individual in amounts sufficient to treat or diagnose human physiological disorders, particularly disorders manifested by aberrant forms and/or abnormal levels of the native E3 ubiquitin protein ligase. The effective amount may vary according to a variety of factors such as the individual's condition, weight, sex and age. Other factors include the mode of administration.

The term "chemical derivative" describes a molecule that contains additional chemical moieties which are not normally a part of the base molecule. Such moieties may improve the solubility, half-life, absorption, etc.of the base molecule. Alternatively the moieties may attenuate undesirable side effects of the base molecule or decrease the toxicity of the base molecule. Examples of such moieties are described in a variety of texts, such as *Remington's Pharmaceutical Sciences*.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve the intended purpose. The determination of an effective dose is well within the capability of those skilled in the art. The therapeutically effective dose can be estimated initially either in cell culture assays, e.g., of neoplastic cells, or in animal models, usually mice, rabbits, dogs, or pigs. The animal model is also used to achieve a desirable concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans. A therapeutically effective dose refers to that amount of protein or its antibodies, antagonists, or inhibitors which ameliorate the symptoms or condition. The exact dosage is chosen by the individual physician in view of the patient to be treated.

Compounds identified according to the methods disclosed herein may be used alone at appropriate dosages defined by routine testing in order to obtain optimal modulation of a human E3 ubiquitin protein ligase biological activity and/or pharmacological activity, while minimizing any potential toxicity. Co-administration or sequential administration of other agents may be desirable.

The pharmaceutical compositions may be provided to the individual by a variety of routes such as subcutaneous, topical, oral and intramuscular. Administration of pharmaceutical compositions is accomplished orally or parenterally. Methods of parenteral delivery include topical, intra-arterial (directly to the tissue), intramuscular, subcutaneous, intramedullary, intrathecal, intraventricular, intravenous, intraperitoneal, or intranasal administration. The present invention also has the objective of providing suitable topical, oral, systemic and parenteral pharmaceutical formulations for use in the novel methods of treatment of the present invention. The compositions containing compounds identified according to this invention as the active ingredient for use in the modulation of physiological conditions can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compounds can be administered in such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection. Likewise, they may also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts. An effective but non-toxic amount of the compound desired can be employed as a E3 ubiquitin protein ligase or variation contemplated herein or human E3 ubiquitin protein ligase modulating agent.

The daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult human/per day. For oral administration, the compositions are preferably provided in the form of scored or unscored tablets containing 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, and 50.0 milligrams of the active ingredient for the symptomatic adjustment of the dosage to the patient to be treated. An effective amount of the drug is ordinarily supplied at a dosage level of from about 0.0001 mg/kg to about 100 mg/kg of body weight per day. The range is more particularly from about 0.001 mg/kg to 10 mg/kg of body weight per day. Even more particularly, the range varies from about 0.05 to about 1 mg/kg. Of course the dosage level will vary depending upon the potency of the particular compound. Certain compounds will be more potent than others. In addition, the dosage level will vary depending upon the bioavailability of the compound. The more bioavailable

and potent the compound, the less compound will need to be administered through any delivery route, including but not limited to oral delivery. The dosages of the modulators desribed herein are adjusted when combined to achieve desired effects. On the other hand, dosages of these various agents may be independently optimized and combined to achieve a synergistic result wherein the pathology is reduced more than it would be if either agent were used alone. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells and conditions.

EXAMPLES

EXAMPLE I

A. Ubiquitin thioester conjugation assay for biological activity

Ubiquitin thiol ester formation by the novel human E3 ubiquitin protein ligase (e.g., SEQ ID NO:3) is determined by change in SDS-PAGE mobility of the E3 ligase band (characteristic of thioester formation between ubiquitin and the protein in this gel-shift assay). Reaction mixtures contain 5-10 ng of recombinant E1 (SEQ ID NO:10 (FIG.12) which is the translated structural coding region of human E1 ubiquitin activating enzyme Uba1), 100 ng of recombinant E2 (SEQ ID NO:11 (FIG.13) which is the translated structural coding region of the E2 ubiquitin conjugating enzyme E217k) [The human version (Ubc2) is preferred as described by Koken, M., *et al.*, PNAS, 88:8865 (1991)], 200 ng of ^{32}P-labeled human E3 ligase (SEQ ID NO:3), and 500 ng of GST-ubiquitin in 20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 4 mM ATP, 10 mM MgCl2, and 0.2 mM dithiothreitol for 3 min at 25 °C. Human E3 ubiquitin protein ligase reactions are terminated by incubating the mixtures for 15 min at 30 °C in SDS-sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.2% bromphenol blue) in the absence of reducing agents and resolved by SDS-PAGE. Radioactively labeled proteins are visualized by autoradiography. Change in the mobility of the E3 ligase band indicates thioester formation in this gel-shift assay. *See, alternate techniques, e.g., Huibregtse, J.M., et al., The HPV-16 E6 and E6-AP Complex Functions as a Ubiquitin-Protein Ligase in the Ubiquitination of p53*, Cell, 75:495 (1995).

EXAMPLE II

Ubiquitination assay for biological activity

Physical interaction between specific E2 enzymes (for example, E217k (SEQ ID NO:11 (structural coding region))) [The human version (Ubc2) is preferred as described by Koken, M., *et al.*, PNAS, 88:8865 (1991)] and the novel human E3 ubiquitin protein ligase (e.g., SEQ ID NO:3) characterizes specific functional cooperativity. This assay employs [^{35}S]Methionine-labeled proteins synthesized in rabbit reticulocyte lysate *in vitro* reactions with a coupled transcription/translation kit (PROMEGA, WI). Kumar, S., *et al.*, J. Biol. Chem., 272:13548 (1997). Messenger RNA is preferred which originates from hematopoietic cells. Five µl aliquots of *in vitro* translated hematopoietic cell mRNA is incubated with 5-10 ng of recombinant E1 (SEQ ID NO:10 (FIG.12) which is the translated structural coding region of human E1 ubiquitin activating enzyme Uba1), approximately 100ng of E2 (E217k (SEQ ID NO:11 (structural coding region))) [The human version (Ubc2) is preferred as described by Koken, M., *et al.*, PNAS, 88:8865 (1991)] (*alternately*, UBC2, UBC3, UBC4, UBC5, UBC6, UBC7, UBC8, UBC9, UBC_{epi}, $UBC_{bendless}$ (as per citations *supra*)), 200 ng of the novel human E3 ubiquitin protein ligase (e.g., SEQ ID NO:3), in 20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 4 mM ATP, 10 mM MgCl2, and 0.2 mM dithiothreitol, for 2 hours at 30 °C. One mg of glutathione-s-transferase (GST)-ubiquitin fusion protein is then added to 5 ml of translation reaction mixture and incubated for an additional 5 min at room temperature before the reaction is quenched with SDS/PAGE loading buffer. Reactions are terminated after 2 h. at 30°C by the addition of SDS-sample buffer. Samples are subject to boiling water heat for 5 min, resolved by SDS-PAGE, and visualized by autoradiography. Samples which contain the ubiquitin fusion protein demonstrate shift in the mobility of protein samples that are ubiquitinated.

EXAMPLE III

Scintillation Proximity Assay (SPA)

Recombinant E1 (using, e.g., SEQ ID NO:10), E2 (using, e.g., SEQ ID NO:11) [The human version (Ubc2) is preferred as described by Koken, M., *et al.*, PNAS, 88:8865 (1991)], and the novel human E3 ubiquitin protein ligase (using, e.g., SEQ ID NO:2) are used to develop a "mix and measure" 96-well SPA (AMERSHAM Scintillation Proximity

Assay) by incorporating ^{125}I-labeled mono-ubiquitin (AMERSHAM) onto a target protein substrate in the presence of ATP and $MgCl_2$. Histone 2A, troponin T, albumin, or α-actin, for example, may be used as target proteins. The ubiquitinated protein is detected using protein A-labeled SPA beads (AMERSHAM) and a polyclonal antibody to the target protein substrate in question. Both protein A-linked and avidin-linked SPA beads have been successfully used in assays using histone2A and biotinylated histone2A, separately, as substrates.

Ubiquitin (UBQ) SPA assay protocol

The reaction mixtures contain 50mM Tris-HCl (pH 7.5), 2mM ATP, 5mM $MgCl_2$, 0.5mM DTT, (5 ng) recombinant E1 (expressed and isolated from SEQ ID NO:10), (10 ng) recombinant recombinant E2 (expressed and isolated from SEQ ID NO:11) [The human version (Ubc2) is preferred as described by Koken, M., *et al.*, PNAS, 88:8865 (1991)], and (20 ng recombinant the novel human E3 ubiquitin protein ligase (expressed and isolated from SEQ ID NO:2), 1μg ^{125}I-Ubiquitin (AMERSHAM, Bucks, UK), and 2μg of biotinylated Histone (SIGMA, St. Louis, MO) to give a final volume of 100ml. Conjugation assays are performed at room temperature for 2 hours. Following incubation, reactions are terminated by addition of 10mM EDTA and 0.1mg/well avidin-linked SPA beads (AMERSHAM).

Final concentrations

	Final Concentration in assay
E1	5ng/μl
E2	10ng/μl
E3	20ng/ul
Radiolabelled UBQ	0.02μCi/well
ATP	2mM
$MgCl_2$	5mM
DTT	0.5mM
Bt-histone	50ng/μl

Stock reagents

1) E1: @ 9.31 mg/ml
2) E2: @ 6.68mg/ml
3) E3: @ 8.60mg/ml
4) ^{125}I-Ubiquitin: @ 0.1μCi/μl
5) ATP: Make at 200mM ie. 110.2 mg/ml in Tris buffer
6) $MgCl_2$: Make at 500mM ie. 101.7 mg/ml in Tris buffer
7) DTT : Make at 1M ie. 154.2 mg/ml in Tris buffer
8)Bt-histone : 2 mg/ml

Buffer: 50mM Tris-HCl pH 7.5

Preparation of reagents

Addition 1 (E1/E2/E3)

(In Tris buffer)

Dilute E1 1:745

Dilute E2 1:267

Dilute E3 1:96

Addition 2 (Label/ATP/MgCl2/DTT/Bt-histone)

Add the following amounts per ml :

(Make up with Tris buffer)

	μl
Label	4
ATP	20
$MgCl_2$	20
DTT	1
Bt-histone	2

Biotinylation of histone2A

Histone2A is biotinylated using BOEHRINGER MANNHEIM kit (Indianapolis, IN (cat. no 1418165)) according to the manufacturers instructions. Briefly, free amino groups of the target protein (histone2A in this case) are reacted with D-biotinyl-e-aminocaproic acid-N-hydroxysuccinimide ester (biotin-7-NHS) by forming a stable amide bond. Nonreacted biotin-7-NHS is separated on a Sephadex G-25 column. The precise molar concentrations used are 4mg Histone2A in 1ml of phosphate buffered saline to which added 20mg/ml biotin-7-NHS is added. the incubation ss carried out at room temperature for 2 hours with gentle shaking.

Stop Mix (Bead/EDTA)

(Make up with Tris buffer)

Add the following amounts per ml :

	µl
Streptavidin SPA Bead	600
500mM EDTA	200

Method

- Use DYNATECH microlite 1 plates
- Add 10µl 300mM EDTA to blank wells
- Add 40µl of Addition 1
- Add 50µl of Addition 2
- Incubate at room temperature for 2 hours
- Add 50µl of Stop Mix
- Leave overnight and count next day

EXAMPLE IV

ELISA assay

Target proteins, e.g., histone2A, are fixed to the bottom of a 96-well ELISA plate in the presence of PEI (polyethylimine). The reaction mix: recombinant E1 (expressed and

isolated from SEQ ID NO:10), recombinant recombinant E2 (expressed and isolated from SEQ ID NO:11) [The human version (Ubc2) is preferred as described by Koken, M., *et al.*, PNAS, 88:8865 (1991)], and recombinant human E3 ubiquitin protein ligase (expressed and isolated from SEQ ID NO:2), mono-ubiquitin (SIGMA), ATP, and $MgCl_2$, is added to each well. Ubiquitinated target protein is detected using a horseradish peroxidase-linked polyclonal antibody to polyubiquitin. Horseradish peroxidase is detected using ATBS (2,2'-azino-di-[3-ethyl-benzthiazoline solfonate]) and ECL (enhanced chemiluminescence) detection systems. This assay may be used as a high throughput screen or as a secondary screen.

1. Coat plate with 100 µl of desired target protein substrate (e.g., histone2A) diluted in PBS (usually at approx. 1-10 µg/ml). Allow to stand overnight at room temperature or 2 hours at 37°C (coated plates may be stored for at least 2 weeks at 4°C).
2. Wash plate 3 times with ELISA Wash Buffer (PBS + 0.05% Tween-20).
3. Add 150 µl of PBS containing 1% BSA to each well. Incubate at room temperature for 2 hours or at 37 degrees for 1 hour.
4. Wash plate 3 times with ELISA Wash Buffer.
5. Add 100 µl of antibody (e.g., Ub N-19, Santa Cruz, Biotechnology, CA) dilutions in PBS containing 1% BSA. Use normal mouse serum as a negative control for ascites and normal rabbit serum as a negative control for rabbit antisera.
6. Cover plate and incubate overnight at room temperature or a minimum of 2 hours at 37°C.
7. Wash plate 3 times with ELISA Wash Buffer.
8. Add 100 µl of the appropriate second antibody enzyme conjugate (e.g., Goat anti-rabbit IgG-HRP) diluted in PBS containing 1% BSA.
9. Cover plate and incubate a minimum of 4 hours at room temperature or 2 hours at 37°C.
10. Wash plate 3 times with ELISA Wash Buffer.

Horseradish Peroxidase (HRP) Substrate (or according to vendor's recommendation)

25 ml 0.1 Citrate-Phosphate buffer, pH5

5 g citric acid monohydrate

7 g Na_2HPO_4 anhydrous

bring volume to 500 ml with dH_2O

Stopping reagent: 6 N H_2SO_4, 50µl/well

Alkaline phosphatase Substrate (or according to vendor's recommendation)

1 tube PNPP (100 mg/ml, 0.2 ml)

20 ml diethanolamine-HCl pH 9.8/1 mM $MgCl_2$

Stopping reagent: 1 M NaOH, 50 µl/well

Add 100 µl of substrate (orthophenyldiamine + substrate buffer + H_2O_2) (6 µl hydrogen peroxide; 10 mg OPD (orthophenyldiamine)); stop reaction when absorbancies in the mid-range of the titration reach about 2.0, or after 1 hour (whichever comes first).

12. Read plate at:

450 nm - HRP unstopped

492 nm - HRP stopped

405 nm - Alkaline phosphatase

(Microplate Spectrophotometer System, CA)

See, Takada, K., *et al.*, Eur. J. Biochem., 233:42 (1995); Takada, K., *et al.*, Biochim. Biophys. Acta., 1290:282 (1996).

EXAMPLE V

Northern blots

Analysis of poly A^+ RNAs from human tissues is generally carried out using a panel of commercially available pre-blotted RNAs (Clontech Laboratories, Palo Alto, CA). Otherwise, Hybond-N^+, supplied by Amersham International PLC, AMERSHAM, Bucks, UK, supported nylon-66 membrane with a pore size of 0.45 microns, is used for the immobilisation of nucleic acids by either UV cross linking or dry heat. Probes are labelled with ^{32}P by random hexamer priming, and hybridisations are carried out in 0.28M sodium phosphate (pH 7.2), 5xDenharts solution, 10% dextran sulphate, 0.1% SDS at 65°C. Membranes are washed to a final stringency of 0.2xSSC,0.1% SDS at 65°C.

Poly A^+ mRNA is prepared directly from ~ $1x10^8$ hematopoietic cells using a FastTrack mRNA isolation kit (INVITROGEN, Carlsbad, CA). Total tissue mRNA is prepared *via* polytron homogenisation in 4M guanidine isothiocyanate, 2.5mM citrate, 0.5% Saccosyl,

100mMb-mercaptoethanol, followed by centrifugation through 5.7M CsCl, 25mM sodium acetate at 135,000g. Poly-A^+ is obtained using FastTrack mRNA isolation kit (INVITROGEN).

SSC

0.15M NaCl + 0.015M sodium citrate pH 7.0

Denhart's reagent

Solution containing 0.02% bovine serum albumin, 0.02% Ficol 400,000 (a non-ionic synthetic polymer of sucrose, dialysed and lyophilised and having an approximate molecular weight of 400,000) and 0.02% polyvinyl pyrrolidone.

EXAMPLE VI

Expression of gst fusion constructs

All E3 ligase GST constructs as well as the control pGEX-5x-3 GST vector alone were transformed into ME DH5α *E. coli* cells. Single colonies were grown overnight at 37 °C shaking at 225 rpm in 10 ml of LB containing 100 μg/ml of ampicillin. The next day, this 10-ml culture was diluted 10-fold to 100 ml in LB/amp and allowed to grow for an additional hour. Protein expression was induced by adding isopropyl-β-D-thiogalactoside (IPTG; Life Technologies, Gaithersburg, MD) to a final concentration of 1 mM and cultured an additional 3 hours. Bacteria were harvested by centrifugation at 5000 rpm for 10 minutes. The bacterial pellet was re-suspended in 15 ml of TBS (50 mM Tris 8.0/150 mM NaCl) containing 0.5% Triton-X100 and 1 mM phenylmethylsulfonylfluoride (PMSF). This suspension was sonicated three times for 20 seconds. After sonication, cellular debris was removed by centrifugation at 10,000 rpm for 10 minutes. Subsequently 150 μl of milk-blocked glutathione sepharose 4B (Pharmacia) was added to the supernatant, and the mixture was rocked at 4 °C for 1 hour. The beads were then collected by centrifugation at 500 x g for 5 minutes and washed three times with 10 ml of the same buffer as used for re-suspension above. Protein concentrations were determined by electrophoresis on an 8% Tris glycine gel and staining with Coomassie blue. Fusion proteins were eluted from the beads by adding of 300 μl of elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl pH 8.0), incubating at room temperature for 10 minutes, and centrifuging at 500 x g for 5 minutes. Elution and centrifugation steps were repeated three times, and the resulting supernatants were pooled.

EXAMPLE VII

Ubiquitination assays

Ubiquination reactions were performed using a protocol based on the published procedure of Hatakeyama, *et al*, J. Biol. Chem., 272:15085 (1997). Reaction mixtures containing 0.5 μg of purified E3 ligase (SEQ ID NO:3) GST fusion protein, 1 μg of 6-His-tagged E2 enzyme (UBCH2 or UBCH7), 1 μg of 6-His-tagged E1 enzyme, 10 μg of bovine ubiquitin (Sigma, St. Louis, MO), and 1 μl of crude lysate from DH5α *E. coli* cells in reaction buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM ATP, 5 mM MgCl2) were incubated at 30C for 2 hours. Reducing sample buffer containing 5% β-mercaptoethanol was added, and samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a purified anti-ubiquitin antibody (Sigma) followed by a purified horseradish peroxidase-conjugated anti-rabbit antibody (Amersham, Bucks, UK) and development using chemiluminescence (ECL; Amersham). Bacterial proteins from the crude lysate served as ubiquination substrates. The FIG.14 results demonstrate that the recombinant human itch E3 ubiquitin ligase has ubiquitinating activity in *in vitro* assays. *See*, FIG.14 → DH5a bacterial lysates · 12% SDS-PAGE · Primary Ab (anti-ubiquitin) · Secondary Ab (Anti-rabbit Ig). The Western blot was developed using the ECL system (Amersham, Bucks, UK).

EXAMPLE VIII

Dominant negative mutant construct

A single amino acid change in the human itchy cDNA sequence, cysteine (TGT) to alanine (GCT), at SEQ ID NO:3 position 820 in the active site was made using the wild-type GST-itchy fusion construct in pGEX-5x-3 as template and the STRATAGENE QuikChange site-directed mutagenesis kit according to manufacturer's instructions. La Jolla, CA. The primers used for the reaction were: upper:

5'-GGCTACCCAGAAGTCATACCGCTTTTAATCGCCTGGACCTGCCAC-3' (SEQ ID NO:14);

lower: 5'-GTGGCAGGTCCAGGCGATTAAAAGCGGTATGACTTCTGGGTAGCC-3' (SEQ ID NO:15). PCR conditions were: 95 °C 30 seconds; 16 cycles of 95 °C 30 seconds, 55 °C 1 minute, and 68 °C 15 minutes; 68 °C 3 minutes. Sequencing of the construct

determined that the amino acid substitution at position 820 was successfully accomplished. *See*, FIG.15.

EXAMPLE IX

Stimulation with PMA and ionomycin

In order to evaluate the role of the SEQ ID NO:3 E3 ligase in the activation of human leukocytes, stimulation experiments were performed independently using peripheral blood mononuclear cells, Jurkat cells, and U937 cells. Peripheral blood monocytes were isolated by layering whole blood diluted 1:1 with PBS onto 1.077 density Ficoll-Hypaque for fractionation and centrifuging at 400x g for 30 minutes. The cells at the interface were isolated, washed two times with PBS, and counted. Approximately 45 x 10^6 cells were used for each timepoint (unstimulated, 2 hours, 8 hours, 24 hours). Jurkat cells and U937 cells were counted, then washed twice with PBS. Approximately 90 x 10^6 cells were used for each timepoint. After determining cells counts, the appropriate number of cells was resuspended in OptiMEM media (Gibco) in 6-well tissue culture dishes. Cells were stimulated with 100 ng/ml PMA and I mM ionomycin for the specified times. After stimulation, cells were collected by low-speed centrifugation and lysed to isolate either protein for Western blot analysis (FIG.16) or RNA for northern blotting using the RNeasy kit (Qiagen, Valencia, CA) (FIG.17).

Western blot analysis (FIG.16)

Approximately 50 x 10^6 jurkat T cells were collected by low speed centrifugation for 5 minutes at room temperature. Cells were washed twice with PBS and resuspended in 100 µl of ice-cold lysis buffer (10 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% Triton-X100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1x COMPLETE protease inhibitors (Boerhinger Mannheim) and put on ice for 15 minutes. Cell debris was removed by centrifugation at 14,000 rpm at 4 C for 10 minutes. Supernatant was subsequently removed to a fresh tube, and protein concentration was estimated based on the number of cells lysed or was determined using the BioRad Protein Assay kit. Lysate aliquots corresponding to the desired protein concentration were mixed with equal volumes of reducing sample buffer containing 5% β-mercaptoethanol and boiled for 5 minutes. The samples were loaded on 10-12% Tris glycine PAGE gels (Novex, San Diego, CA) at 35 mA for approximately 1

hour. Proteins were transferred to nitrocellulose membranes, immunoblotted using an anti-peptide antibody described herein and a horseradish peroxidase-conjugated anti-rabbit secondary antibody (Amersham, Bucks, UK), and developed using Amersham ECL detection reagents and exposing to Hyperfilm-ECL (Amersham). To determine if the human itchy E3 ligase levels change after T cell activation, Jurkat T cells were activated by PMA and ionomycin and protein levels analyzed by Western blot analysis. There was a marked decline in the SEQ ID NO:3 levels 2 h after activation of the jurkat T cells. As activation of T lymphocytes by PMA and ionomycin results in a signal transduction cascade, these findings suggest that the SEQ ID NO:3 'itchy' E3 ligase is involved in turnover of signal transduction proteins in the lymphocytic cells. *See*, FIG.16.

Northern blot analysis (FIG.17)

20 mg total RNA samples were electroporesed on 1% denaturing formaldehyde agarose gels in MOPS buffer (Sambrook, *et al.*, Molecular Cloning, A Laboratory Manual, CSH(1989)) and transferred onto hybond N^+ (Amersham). Probes were labelled with ^{32}P by random hexamer priming, and hybridisations were carried out in 0.28M sodium phosphate (pH 7.2), 5xDenharts solution, 10% dextran sulphate, 0.1% SDS at 65°C. Membranes were washed to a final stringency of 0.2xSSC,0.1% SDS at 65°C. To control loading variations, blots were stripped after autoradiography by boiling in 0.1%SDS, and then rehybridised using a probe containing 1.2kb of a rat glyceraldehyde-3-phosphate dehydrogenase cDNA (GAPDH). Analysis of poly A^+ RNA's from human tissues was carried out using a panel of commercially available pre-blotted RNAs (Clontech, Palo Alto, CA). The results demonstrate that the human itchy E3 ligase mRNA levels dramatically decline within 3 h after stimulation of PBMC's. These results suggest that the 'itchy' E3 ligase gene is involved in turnover of signal transduction molecules in the hematopoietic lineages. *See*, FIG.17.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed

should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

WHAT IS CLAIMED IS

1. A purified polynucleotide comprising a nucleic acid sequence which encodes the polypeptide having the sequence substantially as depicted in SEQ ID NO:3 or a biologically and/or pharmacologically active derivative thereof.

2. The polynucleotide of Claim 1 wherein the polynucleotide sequence comprises the sequence substantially as depicted in SEQ ID NO:2.

3. An expression vector comprising the polynucleotide of Claim 1.

4. An antisense molecule comprising the complement of the polynucleotide of Claim 2 or a biologically-effective portion thereof.

5. A host cell transformed or transfected with the expression vector of Claim 3.

6. A purified polypeptide comprising the amino acid sequence substantially as depicted in SEQ ID NO:3.

7. An antibody specific for the polypeptide of Claim 6.

8. A method for producing cells which express a polypeptide substantially as depicted in SEQ ID NO:3, said method comprising

a) culturing a host cell according to Claim 5 under conditions suitable for the expression of said polypeptide.

9. A method for producing a polypeptide having the amino acid sequence substantially as depicted in SEQ ID NO:3, said method comprising the steps of:

a) culturing a host cell according to Claim 5 under conditions suitable for the expression of said polypeptide, and

b) recovering said polypeptide from the host cell culture.

10. A method of identifying compounds that modulate the biological activity of a E3 ubiquitin protein ligase, comprising:

(a) combining a candidate compound modulator of E3 ubiquitin protein ligase biological activity with a E3 ubiquitin protein ligase polypeptide having the sequence substantially as depicted in SEQ ID NO:3, and

(b) measuring an effect of the candidate compound modulator on the biological activity.

11. A method of identifying compounds that modulate the biological and/or pharmacological activity of a E3 ubiquitin protein ligase, comprising:

(a) combining a candidate compound modulator of E3 ubiquitin protein ligase biological and/or pharmacological activity with a host-cell expressing a E3 ubiquitin protein ligase polypeptide having the sequence substantially as depicted in SEQ ID NO:3, and measuring an effect of the candidate compound modulator on the biological and/or pharmacological activity.

12. A method of treatment of a patient in need of such treatment for a condition which is mediated by the biological and/or pharmacological activity of a E3 ubiquitin protein ligase, comprising administration of a polypeptide substantially as depicted in SEQ ID NO:3 or a biologically and/or pharmacologically active derivative thereof.

13. A method of treatment of a patient in need of such treatment for a condition which is mediated by the biological and/or pharmacological activity of a E3 ubiquitin protein ligase, comprising administration of a nucleic acid substantially as depicted in SEQ ID NO:1 or a biologically-effective derivative thereof.

14. A method of treatment of a patient in need of such treatment for a condition which is mediated by the biological and/or pharmacological activity of a E3 ubiquitin protein ligase, comprising administration of an antisense molecule according to Claim 4 or a biologically-effective derivative thereof.

15. A compound that modulates the biological activity of a E3 ubiquitin protein ligase identified by the method of Claim 10.

16. A compound that modulates the pharmacological activity of a E3 ubiquitin protein ligase identified by the method of Claim 10.

17. A compound that modulates the pharmacological activity of a E3 ubiquitin protein ligase identified by the method of Claim 11.

18. A pharmaceutical composition comprising a compound that modulates the pharmacological activity of a human E3 ubiquitin protein ligase according to Claim 16.

19. A pharmaceutical composition comprising a compound that modulates the pharmacological activity of a human E3 ubiquitin protein ligase according to Claim 17.

20. A method of treatment of a patient in need of such treatment for a condition which is

mediated by the biological activity of a E3 ubiquitin protein ligase comprising administration of a modulating compound according to Claim 16.

21. A method of treatment of a patient in need of such treatment for a condition which is mediated by the biological activity of a human E3 ubiquitin protein ligase, comprising administration of a modulating compound according to Claim 17.

22. A diagnostic composition for the identification of a polypeptide substantially as depicted in SEQ ID NO:3 comprising the antibody of Claim 7.

23. A diagnostic composition for the identification of a polynucleotide sequence substantially as depicted in SEQ ID NO:2 comprised of PCR primers derived from SEQ ID NO:1.

FIG.1

TCGCCGCCGCCCCGAGTCCCGGTACCATGCATTTCACGGTGGCCTTGTGGAGACAACGCCTTAACCCAAGGAAGT
GACTCAAACTGTGAGAACTTCAGGTTTTCCAACCTATTGGTGGTATGTCTGACAGTGGATCACAACTTGGTTCAA
TGGGTAGCCTCACCATGAAATCACAGCTTCAGATCACTGTCATCTCAGCAAAACTTAAGGAAAATAAGAAGAATT
GGTTTGGACCAAGTCCTTACGTAGAGGTCACAGTAGATGGACAGTCAAAGAAGACAGAAAAATGCAACAACACAA
ACAGTCCCAAGTGGAAGCAACCCCTTACAGTTATCGTTACCCCTGTGAGTAAATTACATTTTCGTGTGTGGAGTC
ACCAGACACTGAAATCTGATGTTTTGTTGGGAACTGCTGCATTAGATATTTATGAAACATTAAAGTCAAACAATA
TGAAACTTGAAGAAGTAGTTGTGACTTTGCAGCTTGGAGGTGACAAAGAGCCAACAGAGACAATAGGAGACTTGT
CAATTTGTCTTGATGGGCTACAGTTAGAGTCTGAAGTTGTTACCAATGGTGAAACTACATGTTCAGAAAGTGCTT
CTCAGAATGATGATGGCTCCAGATCCAAGGATGAAACAAGAGTGAGCACAAATGGATCAGATGACCCTGAAGATG
CAGGAGCTGGTGAAAATAGGAGAGTCAGTGGGAATAATTCTCCATCACTCTCAAATGGTGGTTTTAAACCTTCTA
GACCTCCAAGACCTTCACGACCACCACCACCCACCCCACGTAGACCAGCATCTGTCAATGGTTCACCATCTGCCA
CTTCTGAAAGTGATGGGTCTAGTACAGGCTCTCTGCCGCCGACAAATACAAATACAAATACATCTGAAGGAGCAA
CATCTGGATTAATAATTCCTCTTACTATATCTGGAGGCTCAGGCCCTAGGCCATTAAATCCTGTAACTCAAGCTC
CCTTGCCACCTGGTTGGGAGCAGAGAGTGGACCAGCACGGGCGAGTTTACTATGTAGATCATGTTGAGAAAAGAA
CAACATGGGATAGACCAGAACCTCTACCTCCTGGCTGGGAACGGCGGGTTGACAACATGGGACGTATTTATTATG
TTGACCATTTCACAAGAACAACAACGTGGCAGAGGCCAACACTGGAATCCGTCCGGAACTATGAACAATGGCAGC
TACAGCGTAGTCAGCTTCAAGGAGCAATGCAGCAGTTTAACCAGAGATTCATTTATGGGAATCAAGATTTATTTG
CTACATCACAAAGTAAAGAATTTGATCCTCTTGGTCCATTGCCACCTGGATGGGAGAAGAGAACAGACAGCAATG
GCAGAGTATATTTCGTCAACCACAACACACGAATTACACAATGGGAAGACCCCAGAAGTCAAGGTCAATTAAATG
AAAAGCCCTTACCTGAAGGTTGGGAAATGAGATTCACAGTGGATGGAATTCCATATTTTGTGGACCACAATAGAA
GAACTACCACCTATATAGATCCCCGCACAGGAAAATCTGCCCTAGACAATGGACCTCAGATAGCCTATGTTCGGG
ACTTCAAAGCAAAGGTTCAGTATTTCCGGTTCTGGTGTCAGCAACTGGCCATGCCACAGCACATAAAGATTACAG
TGACAAGAAAAACATTGTTTGAGGATTCCTTTCAACAGATAATGAGCTTCAGTCCCCAAGATCTGCGAAGACGTT
TGTGGGTGATTTTTCCAGGAGAAGAAGGTTTAGATTATGGAGGTGTAGCAAGAGAATGGTTCTTTCTTTTGTCAC
ATGAAGTGTTGAACCCAATGTATTGCCTGTTTGAATATGCAGGGAAGGATAACTACTGCTTGCAGATAAACCCCG
CTTCTTACATCAATCCAGATCACCTGAAATATTTTCGTTTTATTGGCAGATTTATTGCCATGGCTCTGTTCCATG
GGAAATTCATAGACACGGGTTTTTCTTTACCATTCTATAAGCGTATCTTGAACAAACCAGTTGGACTCAAGGATT
TAGAATCTATTGATCCAGAATTTTACAATTCTCTCATCTGGGTTAAGGAAAACAATATTGAGGAATGTGATTTGG
AAATGTACTTCTCCGTTGACAAAGAAATTCTAGGTGAAATTAAGAGTCATGATCTGAAACCTAATGGTGGCAATA
TTCTTGTAACAGAAGAAAATAAAGAGGAATACATCAGAATGGTAGCTGAGTGGAGGTTGTCTCGAGGTGTTGAAG
AACAGACACAAGCTTTCTTTGAAGGCTTTAATGAAATTCTTCCCCAGCAATATTTGCAATACTTTGATGCAAAGG
AATTAGAGGTCCTTTTATGTGGAATGCAAGAGATTGATTTGAATGACTGGCAAAGACATGCCATCTACCGTCATT
ATGCAAGGACCAGCAAACAAATCATGTGGTTTTGGCAGTTTGTTAAAGAAATTGATAATGAGAAGAGAATGAGAC
TTCTGCAGTTTGTTACTGGAACCTGCCGATTGCCAGTAGGAGGATTTGCTGATCTCATGGGGAGCAATGGACCAC
AGAAATTCTGCATTGAAAAAGTTGGGAAAGAAAATTGGCTACCCAGAAGTCATACCTGTTTTAATCGCCTGGACC
TGCCACCATACAAGAGCTATGAGCAACTGAAGGAAAAGCTGTTGTTTGCCATAGAAGAAACAGAAGGATTTGGAC
AAGAGTAACTTCTGAGAACTTGCACCATGAATGGGCAAGAACTTATTTGCAATGTTTGTCCTTCTCTGCCTGTTG
CACATCTTGTAAAATTGGACAATGGCTCTTTAGAGAGTTATCTGAGTGTAAGTAAATTAATGTTCTCATTTAGAT
TTATCTCCCAGTGATTTCTACTCAGCGTTTCCAGAAATCAGGTCTGCAAATGACTAGTCAGAACCTTGCTTAACA
TGAGATTTTAACACAACAATGAAATTTGCCTTGTCTTATTCCACTAGTTTATTCCTTTAACAACAATATTTTATG
TGTATCAAAAGTCTCACTTGGGAGTAGTGTTTTTTTTCTTTTAGACATTCTGCAGACATGCAGGGAAGTCCTTTGG
TAACTGCAATATACAAGATTTTCCTATTAAGCCTCTTGGTAAGAGGCATTTGTTAAAAGTGCAAGCTTACTCCTG
CTTCTGGGGATGTGAGCAAAATTCGGGCTTGTGTTCTCCCTCTCATTTTAGTCTGACTTGACTATTGTTTTTCCT
TTCTGGCGCATGAATCCATACATCATTCCTGGAAGTGAGGCAAGACTCTTGCATCTCTACAAAGTAGTTTTGTCA
ATTTGAATTCAGGGAAAAGTTGGTCACAGCCTGCAAATGACTTCATTTGGAAGTCTGATTGTTTCAGTTGCCTGA
CAAATACTACACTTTACAAACAATGTTAACACTGTGATTCCTTCATTGTTTTAAGAAGTTAACCTAGGGCCGGGC
ATGGTGGCTCATACCTGTAATCCTAGCACTCTGGGAGGCCGAGGCAGGAGGATCCCTTTAGCCCAGGAGTTAAAG
ACCAGCCTGGGCAACATAGGGAGACCCTGTCTTTTTTTTGGGCAGCGTGGTGGGGGATAAATAAAWAAAARRAAA
AAAAACKTAGCCTAGAATTAGAATTAATTTAATTGAATTCATCTAAAGATGTCTCTGGTGATTTTTATATGTTCC
GCTATATAATTGATGCTTTATAGTTTTATCATAATCCAACAACTTCAGTTATATTTAATTATTGTTAAGGAGTTT
AAGACTAGAAAGACTAGAGTGCTTTCTAGTCCAAATAGAGGTCAGTGAAACAGCTTTTGACATCAGATTTTCATT
TGAGAGGGAGAGCTGTGGTACTGGCTAAAAAGAAAGGAAGATAACATCCAGTAACCACAGGAATATATTCTCTGT
GAATTAAAAGTCTTCAAAGTTATCATTTCTCTGACATATGTTGGAGTAGTCATTTCCATTCTTTACATTGTCATG
AACTGGATTGATAACCCTCATCTGCAATATTTTCACCCCTAAAATTTTTAACAGGGTTTCCTTTTTTTCTCACGA
CTATTTAAGTTTAGATTGCTCCATTATTAACTGATTAATGCACTTTGAAGTTCTCTGGAATTAATTATTTTAACT
TGGCCTAGCTTCGACTGTCAAGGTGGCTGTTATAAATTTGACTTCATTGGCAGTGGATGAAGCCTAAGCCAGCTG
AGTCTCTATCATAGCTGAACCCTGAGGACAGCCTCATAGCTCATGTATCAGGGACTTTTGCCACATTTCAGAGGC
ATAGCATGAACAAGTAATATTAAGCCAAGAATAAGCAGCAGAACCCTGTTCCATATGGAAAAAAGAAAAACAATT
TTTTGTCCCTAATGTTCTTCCTTTTACATCCTGGAACAACAATAAAAACATTTTTTTAAACTTGTCTACTGTAAG
ATACTGCCATCATAAAGCAGAGACTTACATGAGTGAAAGGGTTGCCTCATCAAGCAGCTCAGTGTAAATGGGGAG
GCTAGGCTCTCCCCAGCCCTATGGTTTTTTTATTTCATGTACCCCAGGAAATACTGTGTGGTTTCTAAAAGCCCT

GGTTGTTAAAAGTAGGGACTCTGCCTTTTTGTTGGTAGGGAGAAAAAACGCTATTGCTTTGTCTTACAGAGCGAA
TGTCTGCCAACTACCCGTTCATTATATAAGTCTGAACTTGGTAATATATGGCTAATGAAGATTAAGCCCTCTATA
AAGACTTCCTGTTGAGGTGAATTCTCATACTGAAATGTAGTTACCTACAATATTTACTAGAGATTTATGAAATTA
AATTAAGAGATAATGTAAGAAAATACATTTTTTTTGGTTCTATATAATGCTTCATGATTCATTTAGGGACCTAGA
AATATTGTGTGAAAATATATAAATATCACCCAAAAGGCTTTCTGCCCTATATTTTTAAAATACAGAATAGTTATA
TTTGAAGTAGCCCTGGCCCTAGTTCTATAGGGCTTGGCTATTTAATATTTTTATGGAAGAAGTGTTTAGTTCTGG
AAAAGGTAAATGCTTGTATATATATTTTTGCAGCCTGGGATCTCCCTACTCCATTTTTTCCTTTAATTTAAGTGG
CCACATGTATATGTCTTCCCTGCTGTGTTAGGAAAATGGGGGCTGGATATCCCAAGAATCAGAGGTTATATAAAA
ATACTGCAAATAGACCGCAGACATAAATATCTACCAAATGCTATCTTAAATTTTGGTCCAAACTGAACATATGGA
AATAGATTTATTGTAAGTATTTACTTAGAGCTTTTTCTTAAATCTGAACTAACTTGCTTTTAGAAGTCTTTTTCT
TTGTAAGCATTGTAAATGCTAATAAATCCTGTTAATTTTTTTTTTTT

FIG.2

ATGGGTAGCCTCACCATGAAATCACAGCTTCAGATCACTGTCATCTCAGCAAAACTTAAGGAAAATAAGAAGAAT
TGGTTTGGACCAAGTCCTTACGTAGAGGTCACAGTAGATGGACAGTCAAAGAAGACAGAAAAATGCAACAACACA
AACAGTCCCAAGTGGAAGCAACCCCTTACAGTTATCGTTACCCCTGTGAGTAAATTACATTTTCGTGTGTGGAGT
CACCAGACACTGAAATCTGATGTTTTGTTGGGAACTGCTGCATTAGATATTTATGAAACATTAAAGTCAAACAAT
ATGAAACTTGAAGAAGTAGTTGTGACTTTGCAGCTTGGAGGTGACAAAGAGCCAACAGAGACAATAGGAGACTTG
TCAATTTGTCTTGATGGGCTACAGTTAGAGTCTGAAGTTGTTACCAATGGTGAAACTACATGTTCAGAAAGTGCT
TCTCAGAATGATGATGGCTCCAGATCCAAGGATGAAACAAGAGTGAGCACAAATGGATCAGATGACCCTGAAGAT
GCAGGAGCTGGTGAAAATAGGAGAGTCAGTGGGAATAATTCTCCATCACTCTCAAATGGTGGTTTTAAACCTTCT
AGACCTCCAAGACCTTCACGACCACCACCACCCACCCCACGTAGACCAGCATCTGTCAATGGTTCACCATCTGCC
ACTTCTGAAAGTGATGGGTCTAGTACAGGCTCTCTGCCGCCGACAAATACAAATACAAATACATCTGAAGGAGCA
ACATCTGGATTAATAATTCCTCTTACTATATCTGGAGGCTCAGGCCCTAGGCCATTAAATCCTGTAACTCAAGCT
CCCTTGCCACCTGGTTGGGAGCAGAGAGTGGACCAGCACGGGCGAGTTTACTATGTAGATCATGTTGAGAAAAGA
ACAACATGGGATAGACCAGAACCTCTACCTCCTGGCTGGGAACGGCGGGTTGACAACATGGGACGTATTTATTAT
GTTGACCATTTCACAAGAACAACAACGTGGCAGAGGCCAACACTGGAATCCGTCCGGAACTATGAACAATGGCAG
CTACAGCGTAGTCAGCTTCAAGGAGCAATGCAGCAGTTTAACCAGAGATTCATTTATGGGAATCAAGATTTATTT
GCTACATCACAAAGTAAAGAATTTGATCCTCTTGGTCCATTGCCACCTGGATGGGAGAAGAGAACAGACAGCAAT
GGCAGAGTATATTTCGTCAACCACAACACACGAATTACACAATGGGAAGACCCCAGAAGTCAAGGTCAATTAAAT
GAAAAGCCCTTACCTGAAGGTTGGGAAATGAGATTCACAGTGGATGGAATTCCATATTTTGTGGACCACAATAGA
AGAACTACCACCTATATAGATCCCCGCACAGGAAAATCTGCCCTAGACAATGGACCTCAGATAGCCTATGTTCGG
GACTTCAAAGCAAAGGTTCAGTATTTCCGGTTCTGGTGTCAGCAACTGGCCATGCCACAGCACATAAAGATTACA
GTGACAAGAAAAACATTGTTTGAGGATTCCTTTCAACAGATAATGAGCTTCAGTCCCCAAGATCTGCGAAGACGT
TTGTGGGTGATTTTTCCAGGAGAAGAAGGTTTAGATTATGGAGGTGTAGCAAGAGAATGGTTCTTTCTTTTGTCA
CATGAAGTGTTGAACCCAATGTATTGCCTGTTTGAATATGCAGGGAAGGATAACTACTGCTTGCAGATAAACCCC
GCTTCTTACATCAATCCAGATCACCTGAAATATTTTCGTTTTATTGGCAGATTTATTGCCATGGCTCTGTTCCAT
GGGAAATTCATAGACACGGGTTTTTCTTTACCATTCTATAAGCGTATCTTGAACAAACCAGTTGGACTCAAGGAT
TTAGAATCTATTGATCCAGAATTTTACAATTCTCTCATCTGGGTTAAGGAAAACAATATTGAGGAATGTGATTTG
GAAATGTACTTCTCCGTTGACAAAGAAATTCTAGGTGAAATTAAGAGTCATGATCTGAAACCTAATGGTGGCAAT
ATTCTTGTAACAGAAGAAAATAAAGAGGAATACATCAGAATGGTAGCTGAGTGGAGGTTGTCTCGAGGTGTTGAA
GAACAGACACAAGCTTTCTTTGAAGGCTTTAATGAAATTCTTCCCCAGCAATATTTGCAATACTTTGATGCAAAG
GAATTAGAGGTCCTTTTATGTGGAATGCAAGAGATTGATTTGAATGACTGGCAAAGACATGCCATCTACCGTCAT
TATGCAAGGACCAGCAAACAAATCATGTGGTTTTGGCAGTTTGTTAAAGAAATTGATAATGAGAAGAGAATGAGA
CTTCTGCAGTTTGTTACTGGAACCTGCCGATTGCCAGTAGGAGGATTTGCTGATCTCATGGGGAGCAATGGACCA
CAGAAATTCTGCATTGAAAAAGTTGGGAAAGAAAATTGGCTACCCAGAAGTCATACCTGTTTTAATCGCCTGGAC
CTGCCACCATACAAGAGCTATGAGCAACTGAAGGAAAAGCTGTTGTTTGCCATAGAAGAAACAGAAGGATTTGGA
CAAGAGTAA

FIG.3

MGSLTMKSQLQITVISAKLKENKKNWFGPSPYVEVTVDGQSKKTEKCNNTNSPKWKQPLTVIVTPVSKLHFRVWS
HQTLKSDVLLGTAALDIYETLKSNNMKLEEVVVTLQLGGDKEPTETIGDLSICLDGLQLESEVVTNGETTCSESA
SQNDDGSRSKDETRVSTNGSDDPEDAGAGENRRVSGNNSPSLSNGGFKPSRPPRPSRPPPPTPRRPASVNGSPSA
TSESDGSSTGSLPPTNTNTNTSEGATSGLIIPLTISGGSGPRPLNPVTQAPLPPGWEQRVDQHGRVYYVDHVEKR
TTWDRPEPLPPGWERRVDNMGRIYYVDHFTRTTTWQRPTLESVRNYEQWQLQRSQLQGAMQQFNQRFIYGNQDLF
ATSQSKEFDPLGPLPPGWEKRTDSNGRVYFVNHNTRITQWEDPRSQGQLNEKPLPEGWEMRFTVDGIPYFVDHNR
RTTTYIDPRTGKSALDNGPQIAYVRDFKAKVQYFRFWCQQLAMPQHIKITVTRKTLFEDSFQQIMSFSPQDLRRR
LWVIFPGEEGLDYGGVAREWFFLLSHEVLNPMYCLFEYAGKDNYCLQINPASYINPDHLKYFRFIGRFIAMALFH
GKFIDTGFSLPFYKRILNKPVGLKDLESIDPEFYNSLIWVKENNIEECDLEMYFSVDKEILGEIKSHDLKPNGGN
ILVTEENKEEYIRMVAEWRLSRGVEEQTQAFFEGFNEILPQQYLQYFDAKELEVLLCGMQEIDLNDWQRHAIYRH
YARTSKQIMWFWQFVKEIDNEKRMRLLQFVTGTCRLPVGGFADLMGSNGPQKFCIEKVGKENWLPRSHTCFNRLD
LPPYKSYEQLKEKLLFAIEETEGFGQE

FIG.4

MGSLTMKSQLQITVISAKLKENKKNWFGPSPYVEVTVDGQSKKTEKCNNTNSPKWKQPLTVIVTPTSKLCFRVWS
HQTLKSDVLLGTAGLDIYETLKSNNMKLEEVVMTLQLVGDKEPTETMGDLSVCLDGLQVEAEVVTNGETSCSEST
TQNDDGCRTRDDTRVSTNGSEDPEVAASGENKRANGNNSPSLSNGGFKPSRPPRPSRPPPPTPRRPASVNGSPST
NSDSDGSSTGSLPPTNTNVNTSTSEGATSGLIIPLTISGGSGPRPLNTVSQAPLPPGWEQRVDQHGRVYYVDHVE
KRTTWDRPEPLPPGWERRVDNMGRIYYVDHFTRTTTWQRPTLESVRNYEQWQLQRSQLQGAMQQFNQRFIYGNQD
LFATSQNKEFDPLGPLPPGWEKRTDSNGRVYFVNHNTRITQWEDPRSQGQLNEKPLPEGWEMRFTVDGIPYFVDH
NRRATTYIDPRTGKSALDNGPQIAYVRDFKAKVQYFRFWCQQLAMPQHIKITVTRKTLFEDSFQQIMSFSPQDLR
RRLWVIFPGEEGLDYGGVAREWFFLLSHEVLNPMYCLFEYAGKDNYCLQINPASYINPDHLKYFRFIGRFIAMAL
FHGKFIDTGFSLPFYKRILNKPVGLKDLESIDPEFYNSLIWVKENNIEECGLEMYFSVDKEILGEIKSHDLKPNG
GNILVTEENKEEYIRMVAEWRLSRGVEEQTQAFFEGFNEILPQQYLQYFDAKELEVLLCGMQEIDLNDWQRHAIY
RHYTRTSKQIMWFWQFVKEIDNEKRMRLLQFVTGTCRLPVGGFADLMGSNGPQKFCIEKVGKENWLPRSHTCFNR
LDLPPYKSYEQLKEKLLFAIEETEGFGQE

FIG.5

SEQ ID NO:4 MGSLTMKSQLQITVISAKLKENKKNWFGPSPYVEVTVDGQSKKTEKCNNT
SEQ ID NO:3 MGSLTMKSQLQITVISAKLKENKKNWFGPSPYVEVTVDGQSKKTEKCNNT

SEQ ID NO:4 NSPKWKQPLTVIVTPTSKLCFRVWSHQTLKSDVLLGTAGLDIYETLKSNN
SEQ ID NO:3 NSPKWKQPLTVIVTPVSKLHFRVWSHQTLKSDVLLGTAALDIYETLKSNN

SEQ ID NO:4 MKLEEVVMTLQLVGDKEPTETMGDLSVCLDGLQVEAEVVTNGETSCSEST
SEQ ID NO:3 MKLEEVVV TLQLGGDKEPTETI GDLSI CLDGLQ LESEVVTNGETTCSESA

SEQ ID NO:4 TQNDDGCRTRDDTRVSTNGSEDPEVAASGENKRANGNNSPSLSNGGFKPS
SEQ ID NO:3 SQNDDGS RSKDE TRVSTNGSDDPEDAGAGENRRVSGNNSPSLSNGGFKPS

SEQ ID NO:4 RPPRPSRPPPPTPRRPASVNGSPSTNSDSDGSSTGSLPPTNTNVNTSTSE
SEQ ID NO:3 RPPRPSRPPPPTPRRPASVNGSPSATSE SDGSSTGSLPPTNTNTNT - -SE

SEQ ID NO:4 GATSGLIIPLTISGGSGPRPLNTVSQAPLPPGWEQRVDQHGRVYYVDHVE
SEQ ID NO:3 GATSGLIIPLTISGGSGPRPLNPVTQAPLPPGWEQRVDQHGRVYYVDHVE

SEQ ID NO:4 KRTTWDRPEPLPPGWERRVDNMGRIYYVDHFTRTTTWQRPTLESVRNYEQ
SEQ ID NO:3 KRTTWDRPEPLPPGWERRVDNMGRIYYVDHFTRTTTWQRPTLESVRNYEQ

SEQ ID NO:4 WQLQRSQLQGAMQQFNQRFIYGNQDLFATSQNKEFDPLGPLPPGWEKRTD
SEQ ID NO:3 WQLQRSQLQGAMQQFNQRFIYGNQDLFATSQSKEFDPLGPLPPGWEKRTD

SEQ ID NO:4 SNGRVYFVNHNTRTTQWEDPRSQGQLNEKPLPEGWEMRFTVDGIPYFVDH
SEQ ID NO:3 SNGRVYFVNHNTRTTQWEDPRSQGQLNEKPLPEGWEMRFTVDGIPYFVDH

SEQ ID NO:4 NRRATTYIDPRTGKSALDNGPQIAYVRDFKAKVQYFRFWCQQLAMPQHIK
SEQ ID NO:3 NRRTTTYIDPRTGKSALDNGPQIAYVRDFKAKVQYFRFWCQQLAMPQHIK

SEQ ID NO:4 ITVTRKTLFEDSFQQIMSFSPQDLRRRLWVIFPGEEGLDYGGVAREWFFL
SEQ ID NO:3 ITVTRKTLFEDSFQQIMSFSPQDLRRRLWVIFPGEEGLDYGGVAREWFFL

SEQ ID NO:4 LSHEVLNPMYCLFEYAGKDNYCLQINPASYINPDHLKYFRFIGRFIAMAL
SEQ ID NO:3 LSHEVLNPMYCLFEYAGKDNYCLQINPASYINPDHLKYFRFIGRFIAMAL

SEQ ID NO:4 FGHKFIDTGFSLPFYKRILNKPVGLKDLESIDPEFYNSLIWVKENNIEEC
SEQ ID NO:3 FHGKFIDTGFSLPFYKRILNKPVGLKDLESIDPEFYNSLIWVKENNIEEC

SEQ ID NO:4 GLEMYFSVDKEILGEIKSHDLKPNGGNILVTEENKEEYIRMVAEWRLSRG
SEQ ID NO:3 DLEMYFSVDKEILGEIKSHDLKPNGGNILVTEENKEEYIRMVAEWRLSRG

SEQ ID NO:4 VEEQTQAFFEGFNEILPQQYLQYFDAKELEVLLCGMQEIDLNDWQRHAIY
SEQ ID NO:3 VEEQTQAFFEGFNEILPQQYLQYFDAKELEVLLCGMQEIDLNDWQRHAIY

SEQ ID NO:4 RH
SEQ ID NO:3 RH

SEQ ID NO:4
SEQ ID NO:3

SEQ ID NO:4
SEQ ID NO:3

FIG.6

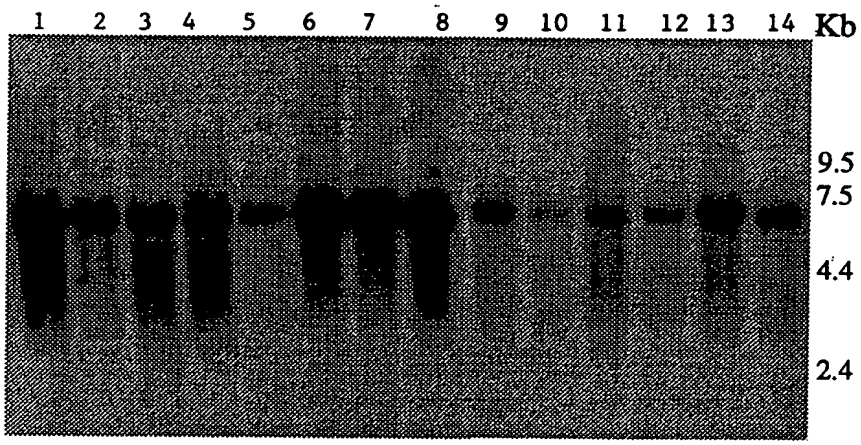

FIG.7

5'- ATGGGTAGCCTCACCATGAAA -3' (SEQ ID NO:5)

5'- TTACTCTTGTCCAAATCCTTC -3' (SEQ ID NO:6)

FIG.8

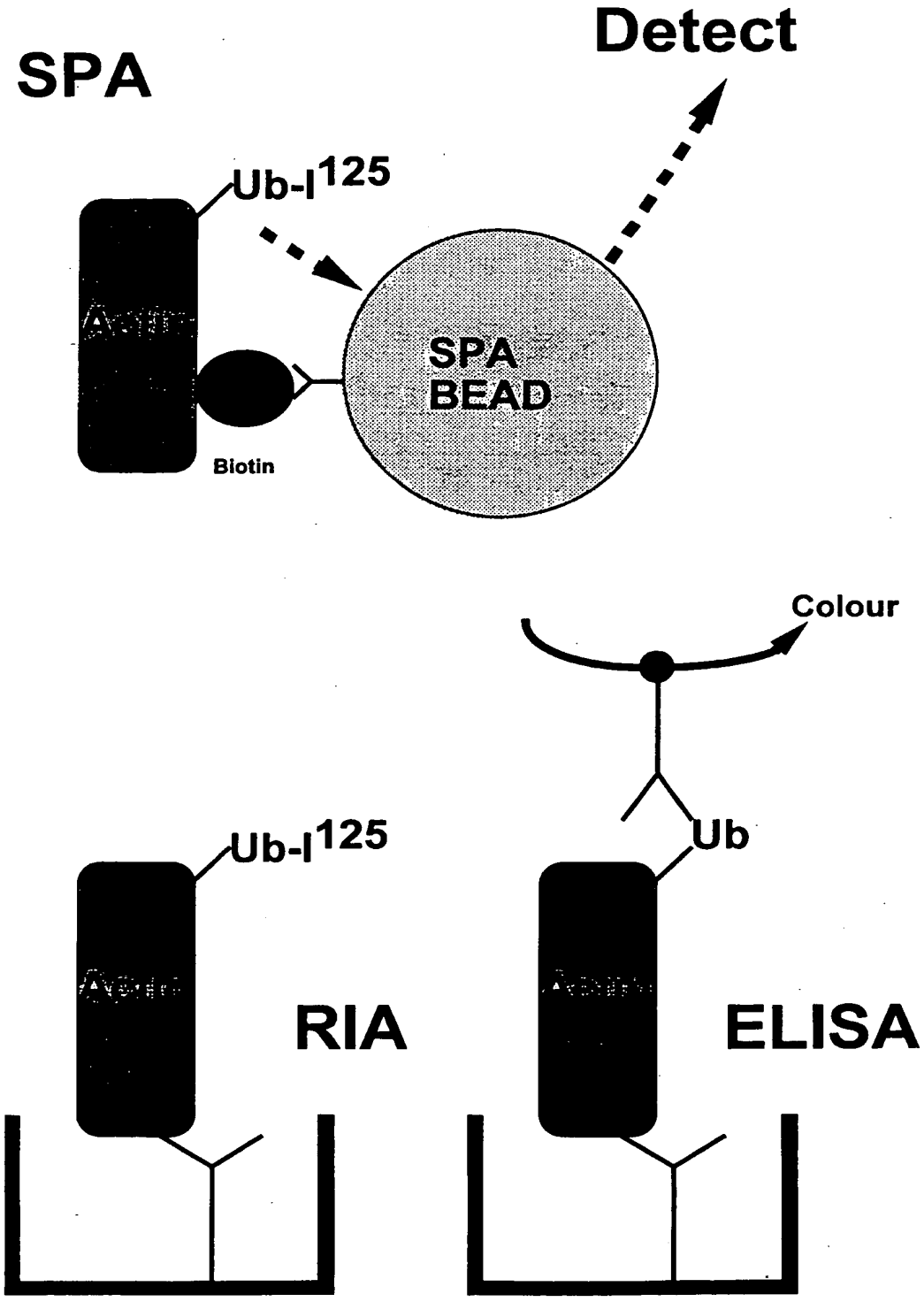
SPA
Detect
Ub-I125
SPA BEAD
Biotin
Colour
Ub
Ub-I125
RIA
ELISA

FIG.9

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FIG.10

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FIG.11

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FIG.12

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FIG.13

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FIG.14

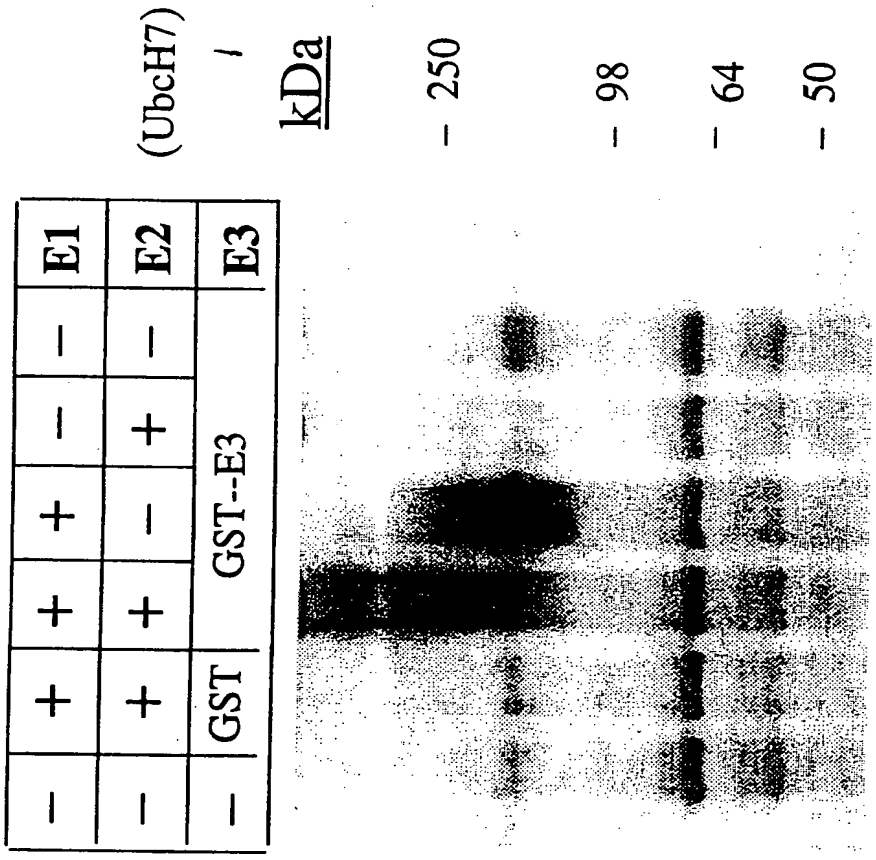

FIG.15

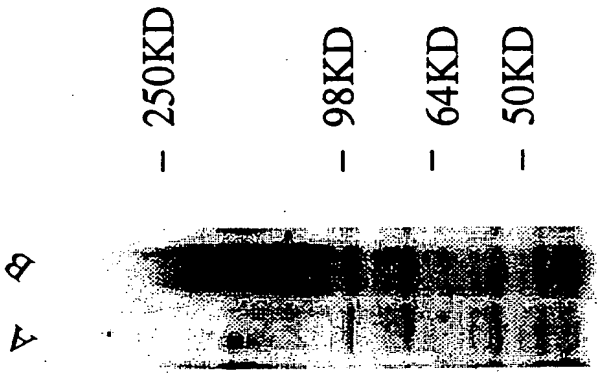

FIG.16

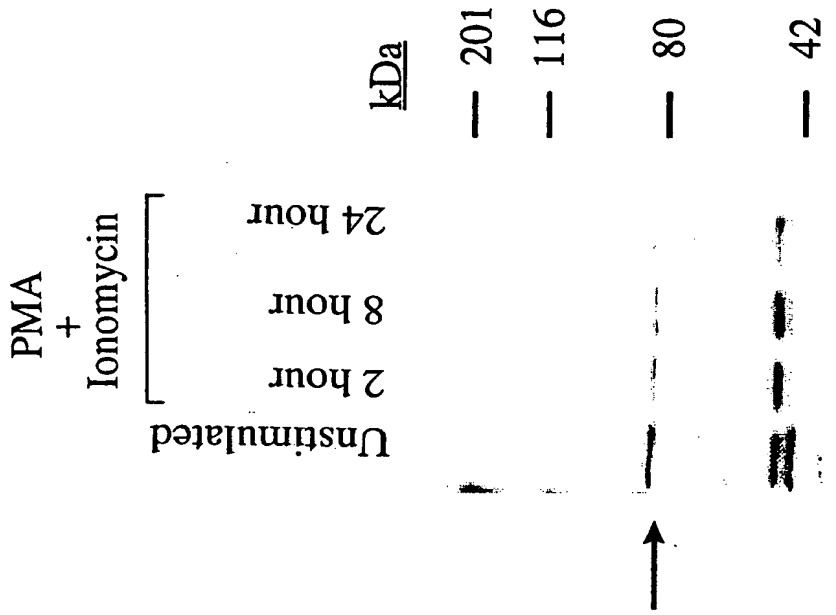
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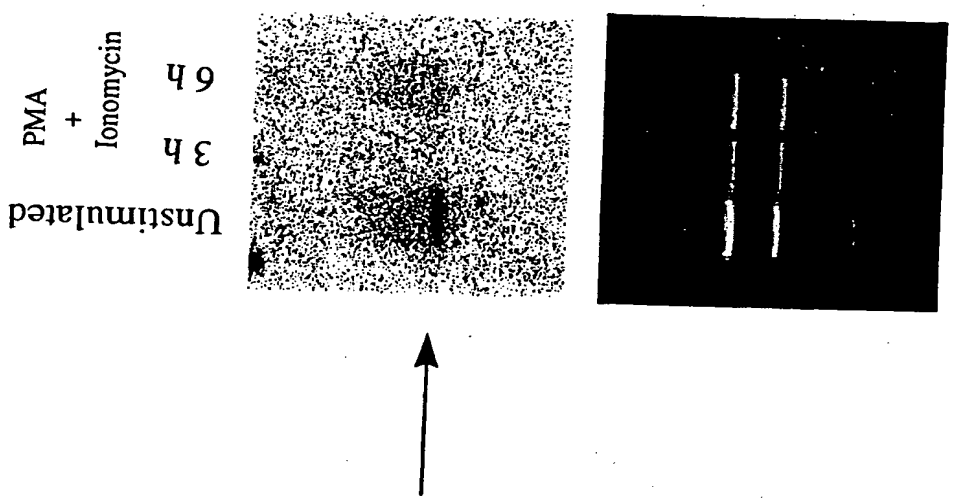
FIG.17



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785 790 795 800
Gly Pro Gln Lys Phe Cys Ile Glu Lys Val Gly Lys Glu Asn Trp Leu
805 810 815
Pro Arg Ser His Thr Cys Phe Asn Arg Leu Asp Leu Pro Pro Tyr Lys
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INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/GB 99/00353

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/52 C12N9/00 C12N5/10 C07K16/40 G01N33/68
A61K38/43 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 37223 A (UNIV NORTH CAROLINA ;CYTOGEN CORP (US)) 9 October 1997 pages 7,10,42-48; 60-65; claims ---	1-3,5-10
X	WO 97 12962 A (COLD SPRING HARBOR LAB ;BEACH DAVID (US); CALIGIURI MAUREEN (US);) 10 April 1997 page 3,8,9,12,22,23-26,35,67; claims ---	1,3-22
X	HILLIER, L., ET AL. : "the WashU-Merck EST project 1997" EMBL SEQUENCE DATA LIBRARY,29 April 1997, XP002103110 heidelberg, germany accession no. AA400315 --- -/--	4

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 May 1999	10/06/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Holtorf, S

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/GB 99/00353

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	PERRY, W.L., ET AL. : "the itchy locus encodes anovel ubiquitin protein ligase that is disrupted in a18H mice" NATURE GENETICS, vol. 18, no. 2, February 1998, pages 143-146, XP002103109 cited in the application cited in the application see the whole document	1-23
A	D'ANDREA, A.D. AND SERHAN, C.N.: "relieving the itch" NATURE GENETICS, vol. 18 , no. 2, February 1998, pages 97-99, XP002103348 cited in the application see the whole document	1-23
A	WEISSMAN A M: "Regulating protein degradation by ubiquitination" IMMUNOLOGY TODAY, vol. 18, no. 4, 1 April 1997, page 189-198 XP004058356 cited in the application	1-23
A	ROLFE M ET AL: "The ubiquitin-mediated proteolytic pathway as a therapeutic area" JOURNAL OF MOLECULAR MEDICINE, vol. 75, no. 3, 1 January 1997, pages 5-17, XP002082283	1-23
P,X	WOOD, J.D., ET AL. : "atrophin-1, the DRPLA gene product, interacts with two families of WW domain-containing proteins" MOLECULAR AND CELLULAR NEUROSCIENCE, vol. 11, no. 3, June 1998, pages 140-160, XP002103111 see the whole document	1-3,5,6, 8,9

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INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/GB 99/00353

Box I Observations where certain claim wer found unsearchable (Continuation of item 1 f first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 12-14 and 20, 21 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter onal Application No
PCT/GB 99/00353

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9737223 A	09-10-1997	AU 2659797 A	22-10-1997
		CA 2250866 A	09-10-1997
		EP 0897541 A	24-02-1999
WO 9712962 A	10-04-1997	EP 0857205 A	12-08-1998